

Biological and Behavioural Markers of Parkinson's Disease

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I dedicate this work to the shattered Muslim Ummah. May you rise to be the shining minaret you once were.

Abstract

Today, upwards of 10 million people—approximately 9 500 of whom reside in New Zealand—are living with Parkinson’s disease (PD). Yet, the means of diagnosing PD remain somewhat similar to those available to James Parkinson in 1817. Recently, however, there has been an increasing interest in the role of biomarkers in PD; these, in turn, are hoped to provide the necessary means by which PD can be diagnosed earlier, treated better and—ultimately—altogether prevented and/or cured.

Given the multifaceted nature of the aetiology underlying PD, a “multi-system” approach to biomarkers is more likely to yield fruitful results. Thus, the overarching aim of this study was to explore several biomarkers (within two realms—biological and behavioural) that may be used at different time-points as the disease progresses.

In the biological markers trials, biofluid samples (i.e., cerebrospinal fluid ‘CSF’ and plasma) were obtained from 11 patients with PD. Analyses of these samples did not detect any blackcurrant anthocyanins either before or after oral supplementation with blackcurrant concentrate for four weeks. Consumption of blackcurrant concentrate, however, significantly increased the CSF concentration of cyclic glycine-proline. This led to the hypothesis of an indirect mechanism underlying the putative benefit of berry-fruit consumption on the risk of developing PD—perhaps through modulating the peripheral resistance to insulin-like growth factor-1 otherwise observed in patients with PD. CSF concentrations of the aminoterminal fragment of C-type natriuretic peptide were significantly lower in PD patients than the reported range from a group of pre-operative orthopaedic patients. Finally, the obtained samples were utilised to characterise the profile of exosomes present in the CSF and plasma of PD patients. The three patients with

the highest plasma exosome concentrations also had the lowest scores on the Montreal Cognitive Assessment.

The behavioural markers study investigated biomarkers in patients with established PD—a stage when cognition may become involved. The emphasis was to obtain an in-depth evaluation of novel eye movement-performance associations. In general, no remarkable differences in eye movement parameters were noted among the three study groups (n = 16 per group): PD with normal cognition (PD-N), PD with mild cognitive impairment (PD-MCI) and matched controls (NC) in natural and laboratory-based neuropsychological tasks. This indicates a relatively preserved organisation of neuropsychological task performance as evident from eye movements among the participants. In addition, some insights into human behaviour on several tasks were gained. In the animal naming task, participants from all three groups tended to fixate on the animal's head in order to name it. Participants also fixated on the distal ends of lines when attempting the Judgement of Line Orientation task. PD-MCI participants were found to make significantly more vertical saccades when searching the Where's Wally?™ Maze task in comparison with NC and PD-N participants. On the Symbol Digit Modalities Test, PD-MCI participants scored significantly lower than NC and PD-N participants. Finally, task organisation of the tea-making task was mostly consistent among the study participants; PD participants (of both groups) executed the task significantly slower than NC participants.

Given the relatively small sample sizes, an exploratory approach was generally taken. To gain confidence in the results of individual findings, further research ought to be carried out in order to exclude the possibility of sampling variability accounting for the reported observations.

Preface

This thesis is submitted as a requirement for the Doctor of Philosophy, part of an intercalated Bachelor of Medicine and Bachelor of Surgery, and Doctor of Philosophy (MBChB/PhD) degree in the Department of Medicine at the University of Otago, Christchurch. The research was carried out at the Van der Veer Clinics and the New Zealand Brain Research Institute (formerly known as the Van der Veer Institute for Parkinson's Disease and Brain Research) under the supervision of Prof. Tim Anderson, Dr Michael MacAskill and Prof. John Dalrymple-Alford. Additionally, Dr Daniel Myall played an ancillary advisory role.

Prof. Anderson provided training for the motor assessment. Prof. Dalrymple-Alford and Mrs Leslie Livingston provided training for the neuropsychological testing. Dr MacAskill designed the software for stimulus-delivery and the simultaneous voice recording. He, with Dr Myall, also guided me through data analysis.

I formulated the hypotheses, and designed the experiments and procedures to test them. I led the data collection and—with the help of collaborators for some experiments (see Supplementary Table 1)—data generation. I also conducted the statistical analysis and interpretation of most of the results.

Various segments of this thesis have formed the basis of two manuscripts under consideration, and nine published articles or letters which are listed below from most to least recent:

1. **Alamri Y**, MacAskill M, Anderson T. Commentary: Aiming for Study Comparability in Parkinson's Disease: Proposal for a Modular Set of Biomarker Assessments to be Used in Longitudinal Studies. *Front Aging Neurosci.* *In press.*

Supplementary Table 1. Collaborators of the biological and behavioural marker studies.

Study	Collaborator(s)	Laboratory	Location	Data collection	Data generation	Statistical analysis
Blackcurrant anthocyanin trial	Dr Sean Connor and Prof. Derek Stewart	The James Hutton Institute	Dundee, Scotland	YA	Collaborators	YA
IGF-1 trial	Mr Dawei Fan and Dr Jian Guan	The Liggins Institute	Auckland, New Zealand	YA	Collaborators	YA
CNP trial	Dr Tim Prickett and Prof. Eric Espiner	Christchurch Heart Institute	Christchurch, New Zealand	YA	Collaborators	YA
Exosome trial	Dr Ben Glossop and Dr Robert Vogel	Izon Science, Ltd.	Christchurch, New Zealand	YA	Collaborators	YA
SDMT task	Ms Maddie Pascoe	New Zealand Brain Research Institute (summer studentship)	Christchurch, New Zealand	YA	Collaborator	Collaborator and YA
Tea-making task	Dr Michael MacAskill	New Zealand Brain Research Institute	Christchurch, New Zealand	YA	Collaborator	Collaborator and YA

2. **Alamri Y**, Vogel R, MacAskill M, Anderson T. Plasma exosome concentration may correlate with cognitive impairment in Parkinson's disease. *Alzheimers Dement (Amst)*. 2016 Aug 29;4:107-108.
3. **Alamri Y**, MacAskill M, Anderson T. Repeated Lumbar Punctures for Non-Clinical Indications: How Do Patients Feel? *Eur Neurol*. 2016 Aug 25;76(3-4):123-124.
4. **Alamri Y**, MacAskill M, Anderson T, Benamer H. Parkinson's Disease in the Gulf Countries: An Updated Review. *Eur Neurol*. 2015;74(3-4):222-5.
5. **Alamri Y**, Al-Busaidi IS, MacAskill M, Anderson T. Anxiety and depression in Parkinson's disease patients in Saudi Arabia: Global neurology. *J Neurol Sci*. 2015 Nov 15;358(1-2):457-8.
6. **Alamri YAS**. Neuroprotection in neurodegenerative disorders: A minireview on an unmet medical need. *Am J Neuroprot Neuroregen*. 2015 Oct;7(1):25-8.
7. **Alamri YA**. Mental health and Parkinson's disease: from the cradle to the grave. *Br J Gen Pract*. 2015 May;65(634):258-9.
8. Espiner E, Dalrymple-Alford J, Prickett TCR, **Alamri Y**, Anderson TJ. C-Type Natriuretic Peptide in Parkinson's Disease: Reduced secretion and response to Deprenyl. *J Neural Transm (Vienna)*. 2014 Apr;121(4):371-8.
9. **Alamri Y**. We are what we eat. Or are we? Early communication of basic research. *NZMSJ*. 2010;11:27-8.

Due to the extended nature of the intercalated MBChB/PhD programme, I have also been involved in a number of projects as a medical student, and later as a House Officer, in addition to the work reported in this PhD thesis. My role in these projects has varied from being the principal researcher to assisting with data collection and analysis, providing technical expertise and reviewing the manuscripts. Some articles are related to the PhD—but are not otherwise described in the thesis—while the others are unrelated. These include 33 published manuscripts and six in press. Below is a chronological list (not including 14 others currently under consideration):

1. **Alamri Y**, Keene A, Pithie A. Acute cystitis caused by commensal *Neisseria oralis*: a case report and review of the literature. *Infect Disord Drug Targets*. *In press*.
2. **Alamri Y**. Successful post-disaster recovery requires adequate pre-disaster preparedness: the case of Gulf countries. *Disaster Med Public Health Prep*. *In press*.
3. **Alamri Y**. The Arabic Hospital Anxiety and Depression Scale: time for a modification? *Chron Respir Dis*. *In press*.
4. **Alamri Y**. Does the choice of religion have an influence on suicidal behaviour? *Acta Psychiatr Scand*. *In press*.
5. **Alamri Y**. Saudi emergency medical services: are resources everything? *Public Health*. *In press*.
6. **Alamri Y**. Dual Degrees in Medicine: Options for Medical Students. *J Cancer Edu*. *In press*.
7. **Alamri Y**, Frizelle F, Al-Mahrouqi H, Eglinton T, Roberts R. Surgical ward round checklist: does it improve medical documentation? A clinical review of Christchurch general surgical notes. *ANZ J Surg*. 2016 Nov;86(11):878-882.
8. **Alamri Y**, Buchwald P, Dixon L, Dobbs B, Eglinton T, McCormick J, Wakeman C, Frizelle FA. Salvage surgery in patients with recurrent or residual squamous cell carcinoma of the anus. *Eur J Surg Oncol*. 2016 Nov;42(11):1687-1692.
9. **Alamri YA**. Is it acceptable for medical professionals to kiss paediatric patients? *J Med Ethics*. 2016 Oct;42(10):636-7.
10. Al-Busaidi IS, **Alamri Y**. Publication rates and characteristics of undergraduate medical theses in New Zealand. *N Z Med J*. 2016 Sep 23;129(1442):46 -51.
11. **Alamri Y**. Journal strict stylistic requirements: Implications for junior researchers. *Free Radic Biol Med*. 2016 Aug;97:618-9.
12. **Alamri Y**, Aboshady OA, Gouda MA. Research output of Arab students. The way forward. *Saudi Med J*. 2016 Sep;37(9):1036.
13. Al-Busaidi IS, **Alamri Y**. Reply: Psychiatric Symptoms in Patients with Stroke: A Six-Month Follow-Up Study. *J Stroke Cerebrovasc Dis*. 2016 Aug;25(8):2089-90.

14. Al-Busaidi IS, **Alamri Y**. Poststroke Anxiety and Depression: Findings from Saudi Arabia. *J Stroke Cerebrovasc Dis*. 2016 Jul;25(7):1653-4.
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16. **Alamri Y**. No seasonal variation is observed in splenic injuries in New Zealand. *Emerg Med Australas*. 2016 Jun;28(3):362.
17. **Alamri Y**, Cockfield A. Peripheral eosinophilia in children with transient synovitis of the hip: 7-year experience from a single centre in New Zealand. *J Child Orthop*. 2016 Jun;10(3):215-8.
18. **Alamri Y**, Al-Busaidi IS. Letter to the Editor: Psychiatric Sequelae of Migraine in Saudi Patients. *J Oral Facial Pain Headache*. 2016 Spring;30(2):171.
19. **Alamri Y**. How do medical student journals fare? A global survey of journals run by medical students. *Educ Health (Abingdon)*. 2016 May-Aug;29(2):136-41.
20. **Alamri Y**. Mental illness in Saudi Arabia: Stigma and acceptability. *Int J Soc Psychiatry*. 2016 May;62(3):306-7.
21. **Alamri Y**, Al-Busaidi IS. Multiple sclerosis in Saudi Arabia: Anxiety, depression and suicidality. *Psychiatry Res*. 2016 Apr 30;238:24.
22. **Alamri Y**. Non-accidental trauma in children presenting with orthopaedic injuries in a single centre in New Zealand. *Australas Med J*. 2016 Apr;9(4):76-77.
23. Ravindran D, **Alamri Y**, Cranefield D. Assessing the response to follow-up recommendations in radiology reports. *N Z Med J*. 2016 Apr 1;129(1432):88-90.
24. **Alamri Y**. Rational recovery: introduction and presence in Australasia. *Australas Med J*. 2016 Mar; 9(3):62-63.
25. **Alamri Y**, Tietjens J, Wakeman C. Dialysis-Associated Telangiectasia: Case Report and Review of the Literature. *Perit Dial Int*. 2016 1-2;36(1):104-105.
26. **Alamri Y**. Uvular amputation through traditional manual uvulectomy. *Ann Trop Med Public Health*. 2016 Jan; 9(3):213-4.
27. **Alamri Y**, Al-Busaidi IS. Anxiety and depression in Saudi patients with epilepsy. *Epilepsy Behav*. 2015 Dec;53:25.

28. **Alamri Y.** Trends in medical student research and publishing. *N Z Med J.* 2015 Jul 3;128(1417):63-4.
29. Al-Busaidi IS, **Alamri Y.** Saving the Survivors. *AMSRJ* 2015; 2(1):118-23.
30. **Alamri Y.** Arab doctors, evolving society and corruption: a medical student's perspective. *Indian J Med Ethics.* 2015 Apr-Jun;12(2):114-5.
31. Monasterio E, **Alamri YA,** Mei-Dan O. Personality characteristics in a population of mountain climbers. *Wilderness Environ Med.* 2014 Jun;25(2):214-9.
32. **Alamri Y,** Soule S, Lim G. Medical image: Cullen's sign. *N Z Med J.* 2013 Dec 13;126(1387):184-6.
33. **Alamri YA.** Gift-giving in the medical student--patient relationship. *J Med Ethics.* 2012 Aug;38(8):506-7.
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35. **Alamri Y,** Al-Mahrouqi H. The USMLE: a guide for New Zealand medical students and graduates. *N Z Med J.* 2012 Jan 25;126(1368):90-2.
36. **Alamri Y.** In person: on shaky ground¹. *Science.* 2011 Sep 30. Available from: http://sciencecareers.sciencemag.org/career_magazine/previous_issues/articles/2011_09_30/caredit.a1100107.
37. **Alamri Y,** Stringer MD. A high-rising epiglottis: a benign anatomical variant. *Clin Anat.* 2011 Jul;24(5):652-4.
38. **Alamri YA.** Herpes rugbiorum: a review on scrum pox and rugby player guidelines. *N Z Med J.* 2011 Jul 8;124(1338):96-9.
39. **Alamri YA.** Rains and floods in Saudi Arabia. Crying of the sky or of the people? *Saudi Med J.* 2011 Mar;32(3):311-3.

I also had the opportunity to present preliminary and complete results from research described in this thesis at several local, national and international conferences. I personally presented five of these presentations (a sixth presentation

¹ Chosen as one of Science Careers' 2011 Best Stories. Available from: <http://www.sciencemag.org/careers/2011/12/science-careers-2011-years-best-stories>.

at the American Academy of Neurology Annual Meeting is awaited in April 2017), and was involved as a co-author for the rest. Below is a chronological list of presentations:

1. **Alamri Y**, MacAskill M, Dalrymple-Alford J, Anderson T. Exploring the World: Gaze Analysis during Visual Search Tasks in Parkinson's Disease. Proceedings of the New Zealand Applied Neurosciences Conference. Neuroepidemiology 2016 Nov;47:143. Auckland, New Zealand 2016.
2. Guan J, **Alamri Y**, Fan D, MacAskill M, Anderson T. Cyclic glycine-proline increased in the cerebrospinal fluid in Parkinson's patients after supplementation of blackcurrant anthocyanins: Potential biomarker for treatment. Mov Disord. 2016 Jun;31(S2):S284. Berlin, Germany 2016.
3. **Alamri Y**, MacAskill M, Myall D, Dalrymple-Alford J, Anderson T. Animal-naming in patients with Parkinson's disease. Society for Neuroscience Conference, poster N5.431.10. San Diego, USA 2013.
4. MacAskill M, **Alamri Y**, Myall D, Dalrymple-Alford J, Anderson T. Tea with milk and sugar: Gaze and action coordination in a real-world task in Parkinson's disease. In K. Holmqvist, F. Mulvey & R. Johansson (Eds.), Book of Abstracts of the 17th European Conference on Eye Movements. Journal of Eye Movement Research, 6(3), 512. Lund, Sweden 2013.
5. Prickett TCR, **Alamri Y**, Anderson TJ, Dalrymple-Alford JC, Espiner EA. Putative Neuroprotective Role for C-type Natriuretic Peptide (CNP) in Humans: Monoamine Oxidase Inhibition (MAOI) Prevents Decline in CSF NTproCNP in Parkinson's Disease (PD). Endocrine Society's 95th Annual Meeting Conference proceedings. San Francisco, USA 2013.
6. **Alamri Y**, MacAskill M, Anderson T. Do blackcurrant metabolites cross the blood-brain barrier in patients with Parkinson's disease? Otago Postgraduate Conference, poster 2.1. Christchurch, New Zealand 2010.
7. **Alamri Y**, MacAskill M, Anderson T. Do blackcurrant metabolites cross the blood-brain barrier in patients with Parkinson's disease? Proceedings of the 28th Australasian Winter Conference on Brain Research, 28, poster 4.26. Wanaka, New Zealand 2010.

8. **Alamri Y**, MacAskill M, Anderson T. The use of blackcurrants in patients with Parkinson's disease. Health Research Society of Canterbury Expo., poster 1.7. Christchurch, New Zealand 2010.

In addition, I have been fortunate to be involved in several other research-related activities, especially in the latter phase of the programme. I have been involved in the Advisory Board of the New Zealand Medical Student Journal (NZMSJ) and serve as an *ad hoc* reviewer for the journals PLoS One and BMJ Open.

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As a consequence of the protracted nature of the intercalated MBChB/PhD programme, time dedicated to my PhD research was fragmented. This invariably led to an extended course of study during which I relied upon the assistance of many people.

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Leaving home for New Zealand to pursue my tertiary studies was daunting for the then 17-year-old me. My journey was made a great deal easier and more enjoyable, however, by meeting friends whom I regard as brothers. Dr Alwalaa Althagafi, Dr Amr Alwakeel, Dr Amr Binsadiq, Dr Danus Ravindran, Dr Khalid Alsahli and Dr Rashid Alaraimi: thank you for your support. In Christchurch, I would like to thank Dr Ahmed Al-Salmi, Dr Hussain Allawati, Dr Mahmoud Alkhater, Dr Rashid Almandhari and Dr Sulaiman AlMubarak. I am also very grateful to Dr Ibrahim S Al-Busaidi for agreeing—on short-notice—to offer a critical pair of eyes to the introductory chapters of this thesis, and to Mr Maxmoud Osman for his help with the reference list. Dr Haitham Al-Mahrouqi and Dr Sultan Al-Shaqsi deserve a special acknowledgement for being an inspiration and role models to whom one looks up in awe and admiration.

As part of my combined programme, I had to complete my clinical years mid-way through my PhD research. I am incredibly grateful to the Christchurch School of Medicine class of 2014, who made me feel one of their own. I am especially appreciative of the support and encouragement of Dr William Angus. Moreover, during my medical elective at UCLA in the United States, I had the privilege of meeting collaborators Dr Fahad Alnemary and Dr Faisal Alnemary; your drive and passion for neurodevelopmental research is nothing short of admirable.

The administrative staff at the Christchurch School of Medicine in general, and the Department of Medicine in particular, has been exceptional. Postgraduate students here are provided with such a healthy and supportive environment, it is hard to be matched elsewhere in New Zealand. In particular, I would like to

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Having reached this milestone of my formal education, I would like to thank an unsung hero—my uncle Matar Al-Zahrani. Throughout the years, you kept a keen interest in my education and aspirations. You encouraged me to reach my full potential and be a nidus for positive change. For this, I thank you unreservedly.

To my family, I am sorry my sojourn has been too long. Mum and Dad—thank you for your continual and unconditional love, support and prayers. I would not be where I am today without you. I hope I made you proud. To my younger siblings: Rahaf, Raghad, Ammar and Hammodi, I hope your big brother's journey has inspired you. I am confident we will be celebrating your many successes in the near future.

To Lannah, you are the bliss of Daddy's existence. Your arrival to this world has added immense happiness and meaning to Mummy and Daddy's lives. Seeing you grow before our eyes is nothing short of pure joy. We look forward to seeing you grow into a successful confident Muslim woman.

Finally, to the love of my life, Sarah: you are as beautiful on the inside as you are outside. Thank you for your limitless love, for believing in me and for your patience while I disappeared into الخن for hours on end. I look forward to spending more time with you and Lannah.

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List of Abbreviations

AD	Alzheimer's disease	MitoQ	Mitoquinone
ALS	Amyotrophic lateral sclerosis	MMP ⁺	1-methyl-4-phenylpyridinium
ANOVA	Analysis of variance	MMSE	Mini Mental State Examination
ANP	Atrial natriuretic peptide	MoCA	Montreal Cognitive Assessment
A β	Amyloid- β	MPDP ⁺	1-methyl-4-phenyl-2,3-dihydropyridinium
BBB	Blood-brain barrier	MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
BBS	Brain-barrier system	MR	Matrix reasoning
BCB	Blood-CSF barrier	MRI	Magnetic resonance imaging
BNP	B-type natriuretic peptide	MS	Multiple sclerosis
C3G	Cyanidin 3-O- β -glucoside	mTorr	millitorr
C3R	Cyanidin 3-O- β -rutinoside	NEP-1	Neutral endopeptidase (or neprilysin)-1
CD63	Cluster of differentiation antigen 63	NPR	Natriuretic peptide receptors (types A, B or C)
cGMP	Cyclic guanosine monophosphate	NTA	Nanoparticle tracking analysis
cGP	Cyclic glycine-proline	NT-CNP-53	Aminoterminal fragment of CNP-53
CNP	C-type natriuretic peptide	NTproCNP	Aminoterminal fragment of proCNP
CNS	Central nervous system	NZ	New Zealand
CoQ10	Coenzyme Q ₁₀	NZBRI	New Zealand Brain Research Institute
cVA	Corrected visual acuity	OKN	Optokinetic nystagmus
CVOs	Circumventricular organs	OS	Oxidative stress
D3G	Delphinidin 3-O- β -glucoside	PD	Parkinson's disease
D3R	Delphinidin 3-O- β -rutinoside	PD-D	Parkinson's disease with dementia
DATATOP	Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism	PD-MCI	Parkinson's disease with mild cognitive impairment

dIPFC	Dorsolateral prefrontal cortex	PD-N	Parkinson's disease with normal cognition
DNA	Deoxyribonucleic acid	pH	Potential of hydrogen
DNP	Dendroaspis natriuretic peptide	RNA	Ribonucleic acid
ECF	Extracellular fluid	ROS	Reactive oxygen species
EGC	Epigallocatechin	RPM	Revolutions per minute
FEF	Frontal eye field	SC	Superior colliculus
GC	Guanylyl cyclase	SD	Standard deviation
G _c	Crystallised intelligence	SDMT	Symbol digit modalities test
G _f	Fluid intelligence	SEF	Supplementary eye field
GP _e	External globus pallidus	SMI	SensoMotor Instruments
HADS	Hospital Anxiety and Depression Scale	TBI	Traumatic brain injury
HD	Huntington's disease	TRPS	Tunable resistive pulse sensing
IGF	Insulin-like growth factor	UPDRS	Unified Parkinson's Disease Rating Scale
IGFBP	Insulin-like growth factor-binding protein	US	United States
JLO	Judgement of line orientation	US	United States
LP	Lumbar puncture	VOR	Vestibulo-ocular reflexes

CHAPTER 1

Introduction and Rationale for the Biomarkers Studies

1.1 Parkinson's disease

1.1.1 Clinical definition

Parkinson's disease (PD) is a degenerative disorder that primarily affects the nervous system. Whilst the pathophysiology underlying idiopathic PD has long been elusive to pinpoint, mounting evidence is implicating neuroinflammation as a central precipitating and perpetuating culprit (i.e., Braak's hypothesis; Barnum & Tansey, 2012).

In the central nervous system (CNS), this neuroinflammatory process manifests as neuronal degeneration particularly involving the dopamine-releasing cells (i.e., dopaminergic neurons); and when a patient starts exhibiting symptoms of PD, 50 to 70% of these dopaminergic cells will have been lost, with a resultant reduction in dopamine neurotransmitter stores of about 80% (Fearnley & Lees, 1991). This state of 'dopamine deficiency' is the basis of the motor symptoms experienced by PD patients. Non-motor symptoms (e.g., constipation and mood disorders; see review by Alamri, 2015 for the latter) are likely the result of degenerative processes extending to other non-dopaminergic systems (e.g., cholinergic pathways; Chaudhuri, Healy, & Schapira, 2006).

Even though there are a number of diagnostic criteria for PD (e.g., Queen Square Brain Bank clinical diagnostic criteria, Hughes, Daniel, Kilford, & Lees, 1992; and the Movement Disorders Society clinical diagnostic criteria, Postuma et al., 2015), there are essentially three cardinal features of PD: muscular rigidity, resting tremor and bradykinesia. Postural instability is also a common feature, but does not present until later in the course of the disease, and was hence deemphasised in the clinical criteria by Postuma *et al* (2015). Each of these symptoms varies in its time of onset, severity and rate of progression along the course of the disease. Figure 1-1 shows a schema of

PD subtypes according to the initial presentation of the patient (Leventoglu & Baysal, 2008).

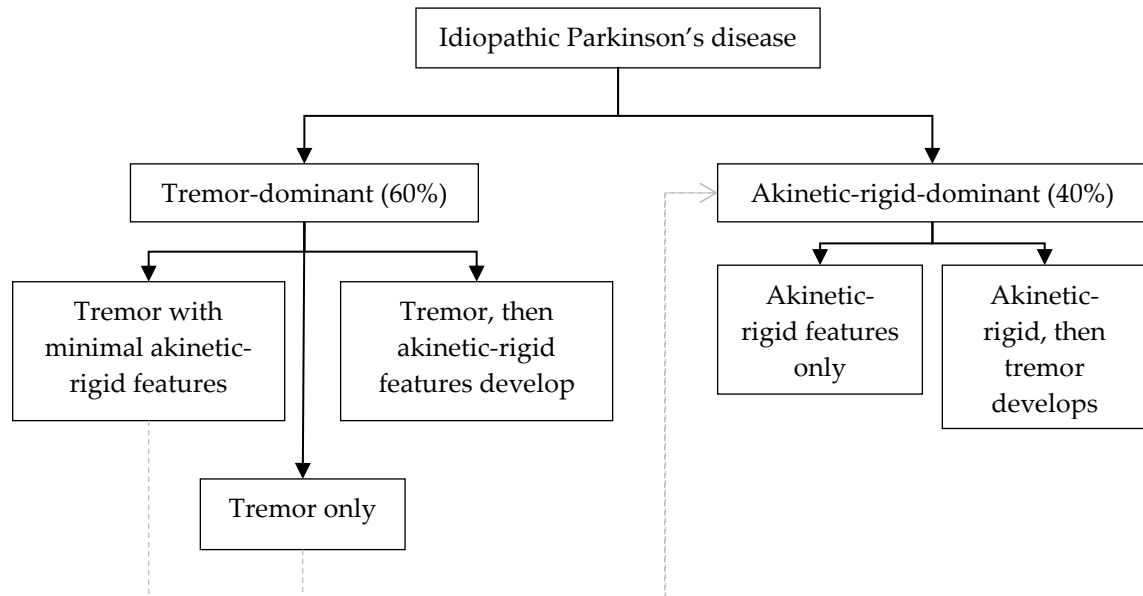


Figure 1-1. Classification of idiopathic Parkinson's disease (PD) according to Leventoglu and Baysal (2008). Whilst useful to highlight the clinical heterogeneity of the initial presentation of idiopathic PD, the classification remains controversial as most patients possibly eventually converge to an akinetic/rigid-type.

Muscular rigidity is defined as the resistance that muscles exert to passive movement across all ranges (Jankovic, 2008)—this form of rigidity exhibited by PD patients is referred to as “lead-pipe” rigidity. When lead-pipe rigidity is combined with parkinsonian tremor, it is termed “cogwheel” rigidity. It is sometimes accompanied by joint stiffness, joint pain or both, and these symptoms sometimes constitute the earliest complaints in PD patients (Riley, Lang, Blair, Birnbaum, & Reid, 1989).

Resting tremor, also called parkinsonian tremor, is characterised by slow (4-6 cycles per second), involuntary rhythmic muscle movements that occur at rest, and is nearly always more prominent in the distal part of a limb (Jankovic, 2008). However, it can also involve the lips, tongue, jaw, chin and trunk, but rarely the head, neck or voice (Jankovic, 2008; Lees, Hardy, & Revesz, 2009). It may be described as ‘pill-rolling’ in nature, pointing to its resemblance to the voluntary manipulation of small objects or

pills with fingers (Elble, 2008). It usually starts unilaterally, and is one of the most easily identifiable symptoms of PD (Jankovic, 2008).

Bradykinesia refers to the slowness of movements. PD is, by far and away, the most common cause of bradykinesia (Jankovic, 2008; Lees et al., 2009). Newly-diagnosed PD patients have been shown to have a prolongation of reaction times upon motor-task testing. This indicates that the slowness is not only related to executing the movement, but also to planning it—a feature sometimes referred to as “bradyphrenia” (Cooper, Sagar, Tidswell, & Jordan, 1994). In addition, bradykinesia was found to be the cardinal feature that best correlates with the extent of dopamine deficiency in PD patients. It is, hence, the most characteristic and important of the three cardinal features (Lees et al., 2009; Vingerhoets, Schulzer, Calne, & Snow, 1997), and the only necessary one—along with at least one of tremor or rigidity—for diagnosis (Postuma et al., 2015).

One of the later manifesting symptoms of PD is postural instability which is chiefly due to the loss of postural reflexes. Such instability leads to frequent falls, loss of confidence and secondary traumatic injuries (Williams, Watt, & Lees, 2006).

1.1.2 Epidemiology

1.1.2.1 General remarks

PD is the second most common neurodegenerative disease in the elderly after Alzheimer’s disease (AD; Mehta et al., 2007). Since PD is an age-related disorder, it is expected that PD incidence will increase in the future, as there are far more people surviving beyond 65 years of age than there ever used to be (Joseph et al., 1999). This increase may not be continuous, however, as PD incidence may start to decline in persons surviving past 90 years of age (see below).

Even though PD is often quoted to have a global prevalence rate of 0.1%, this figure increases substantially when populations are age-stratified (Tanner, 2003). For example, Tanner (2003) estimated that PD affects around 3% of persons over 65 years and 4-5% of people over 85 years, while patients under 40 year of age represent only 5 to 10% of all PD patients. As evident from the literature, prevalence rates can vary considerably according to, for example, the diagnostic criteria used in a particular study or the methodology of screening. Although the studies published in the literature on the epidemiology of PD are aplenty, it was of interest to me to examine some of the reported prevalence rates of PD in Australasia and the Arab World.

1.1.2.2 Australasia

In the latest of studies looking at the prevalence of PD in Australian populations, Mehta *et al* reported PD prevalence rate to be around 460 per 100 000 persons 49 years or older (2007). This rate increased to as high as 820 per 100 000 in persons aged 70 to 79 years, but declined again to 560 per 100 000 in persons aged 80 year and over. Other reported prevalence rates (not age-stratified) varied according to the community studied. For example, the estimated prevalence of PD in urban Queensland was 145 per 100 000 persons (Hely et al., 1999), meanwhile rural Queensland was reported to have an estimated prevalence of 415 per 100 000 persons (McCann et al., 1998).

Studies from New Zealand have been less numerous. In 1962, the prevalence of PD was examined in Wellington's residents. This was estimated to be 106 per 100 000 persons (Pollock & Hornabrook, 1966). Almost 30 years later, a similar cross-sectional study of Dunedin's population was conducted and showed a PD prevalence of 91 per 100 000 persons aged 55 to 64 years and 476 per 100 000 persons aged 65 to 74 years (Caradoc-Davies, Weatherall, Dixon, Caradoc-Davies, & Hantz, 1992). In contrast to

the decline in prevalence seen in Australian population aged 80 years or more, the prevalence in the Dunedin study kept increasing to reach 1 995 per 100 000 persons 84 years or older. However, this New Zealand study is more than 25 years old now and originates from a single city in New Zealand.

More recent national estimates from New Zealand started appearing in the literature. In 2013, the age-standardised prevalence rate of PD was 245 per 100 000 persons in New Zealand Europeans (also known as Pākehā), and 104 per 100 000 person in New Zealand Māori (Pitcher et al., 2016). In the ageing population, the prevalence of PD peaked at 2 200 per 100 000 in persons aged 85-89, gradually decreasing to 640 per 100 000 in the 100+ age-group (Myall et al., 2016). These latter findings are more or less in line with trends observed in Australia (Mehta et al., 2007).

1.1.2.3 Arab World

Arabic-speaking countries, sometimes referred to as the Arab World, stretch over two continents (i.e., Asia and Africa); they include countries from Oman in the East to Morocco and Mauritania in the West. Arab families tend to be large units with a high rate of consanguineous marriage, raising the risk of genetic disorders (Tadmouri et al., 2009). With the help of globalisation and oil-discovery, there has been a recent improvement in health services in the Arab World (Benamer, de Silva, Siddiqui, & Grosset, 2008). This accounts for the expected increase in life-expectancy, invariably leading to an increase in the incidence of PD as the population ages.

Only a handful of studies have looked at the prevalence of PD specifically. In a Libyan population, PD had a prevalence rate of 285 per 100 000 persons aged more than 50 years (Ashok, Radhakrishnan, Sridharan, & Mousa, 1986). Another study from Saudi Arabia reported a prevalence rate of 27 per 100 000 persons (al Rajeh et al., 1993). Compared with Australasian populations, these prevalence rates—especially in

Saudi Arabia — are much lower, even after accounting for the differences in national age averages and life expectancies (Alamri, MacAskill, Anderson, & Benamer, 2015).

Even within the Arab World, North African Arabs seem to have a much higher prevalence rate of PD than those that of other Arab populations (reviewed by Alamri et al., 2015). This may be attributable to a relatively large contribution of genetic cases of PD (e.g., *LRRK2* G2019S mutations) in Arabs of North African origin (Benamer & de Silva, 2010). However, the comparability of the results in Arab populations may be limited since these studies were conducted two to three decades ago.

1.2 Biomarkers in PD

1.2.1 Definitions and characteristics

Various definitions exist in the literature for biomarkers. In medicine, a biomarker may refer to an indicator upon which an inference about the person's health can be made. The National Health Institute's Biomarkers Definitions Working Group (2001) defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention".

A biomarker need not necessarily be a *direct* indicator for a disease process. In other words, a biomarker may act as a substitute for an often difficult-to-quantify clinical end-point. For example, a general practitioner may monitor the patient's blood pressure to get an estimate of their risk of developing a cerebrovascular accident (Hansson et al., 1998). This subset of biomarkers is appropriately termed surrogate biomarkers. Whilst not always ideal, they often provide easy and useful proxies for

the disease in question, and are based on epidemiological, pathophysiological, therapeutic or other scientific evidence.

The ideal biomarker is simple to obtain, validated by rigorous studies and inexpensive. In fact, Michell and colleagues (2004) suggested eight characteristics for the ideal biomarker for disease “X”:

1. Close (first-degree) association with X without relying on intermediate variables, thereby minimising the risk of dissociation.
2. It must sensitively reflect even small changes in X.
3. Treatment has no direct effect on the biomarker; it only changes with a true change in X.
4. The biomarker changes linearly (either negatively or positively) in response to a change in X.
5. Measurements are reproducible at a different time or in a different centre.
6. The biomarker should ideally capture all changes in X so that no information is lost.
7. The optimal clinical biomarker should be cheap, non-invasive and quick to measure by untrained staff.
8. Appropriately thorough validation of the above (depends upon the use of the biomarker and implications of error).

Evidently not many biomarkers—and none in the case of PD (see discussion below)—fulfil all these criteria. Still, available biomarkers in other diseases have proved useful for disease prevention, early diagnosis, disease monitoring, therapeutic target identification and assessment of drug response. A single biomarker is unlikely to provide all pertinent information, and often multiple biomarkers of different types are employed in patient care.

1.2.2 Classification of biomarkers

There are many methods by which biomarkers have been classified and typified in the literature—even more numerous than there are biomarker definitions. Trying to capture all classifications of biomarkers in the literature can be overwhelming. However, ordering the different biomarkers into categories and types can make the task more manageable. A classification scheme is suggested below.

At the most basic level, biomarkers pertaining to a particular disease process can be categorised according to the disease stage. In asymptomatic persons, biomarkers may be pre-pathological (e.g., screening the population at risk), pre-symptomatic (i.e., persons with a confirmed disease process who have not yet displayed any symptoms) and pre-clinical (i.e., patients whose symptoms are so mild that they do not warrant visiting the doctor).

Once patients manifest symptoms, the purpose of biomarkers shifts. They can now be utilised to confirm the patient's diagnosis (i.e., diagnostic biomarkers), monitor disease progression (i.e., progress biomarkers), evaluate response to treatment and sometimes monitor levels of medication (i.e., monitoring biomarkers).

Finally, especially if the disease is incurable, patients may then be interested in their outlook (i.e., prognostic biomarkers). Towards the terminal stages of their illness, patients may be offered comfort cares (i.e., 'palliative' and 'end-of-life' biomarkers become applicable).

At a higher echelon, the basic categories of disease biomarkers can be divided into several types. In other words, several modalities (e.g., clinical examination, blood tests or imaging studies) may be employed as biomarkers for each stage of the patient's journey with the illness. Findings on one *type* of biomarker (e.g., an imaging

study) may differ as the patient progresses from one *category* of disease (e.g., pre-pathological) to another (e.g., diagnostic).

Starting at the patient's bedside, clinical examination manoeuvres are routinely elicited by clinicians in an attempt to determine the patient's disease stage. Pen-and-paper neuropsychological tests may also be administered. Physiological testing (e.g., electromyography) is sometime quintessential to making certain diagnoses and monitoring progress or resolution. Biochemical analyses may then be performed by obtaining biological fluid 'biofluid' samples (e.g., blood or cerebrospinal fluid, 'CSF') from the patient; they can also be obtained from a larger healthy sample (e.g., genetic testing of a family or population screening studies). The patient may also need to undergo specialised radiological imaging or functional (e.g., eye movement) assessments. Finally, post-mortem examination may also be undertaken to elucidate the exact cause of demise or evaluate the presence of co-pathologies (e.g., concomitant AD and PD; Hughes, Daniel, Blankson, & Lees, 1993).

1.2.3 Biomarkers for PD

1.2.3.1 Rationale

There has been an increasing interest in finding biomarkers for PD; this is evident from the number of "Parkinson's disease biomarker" articles indexed by the MEDLINE® database over the years (see Figure 1-2). Given the complexity underpinning PD's pathological processes, a useful single biomarker is unlikely to encompass the plethora of disease facets. However, the diverse features of PD's aetiological process and manifestations make it particularly suited to have a number of biomarkers (of different categories and types) that serve distinct purposes at various stages of the disease.

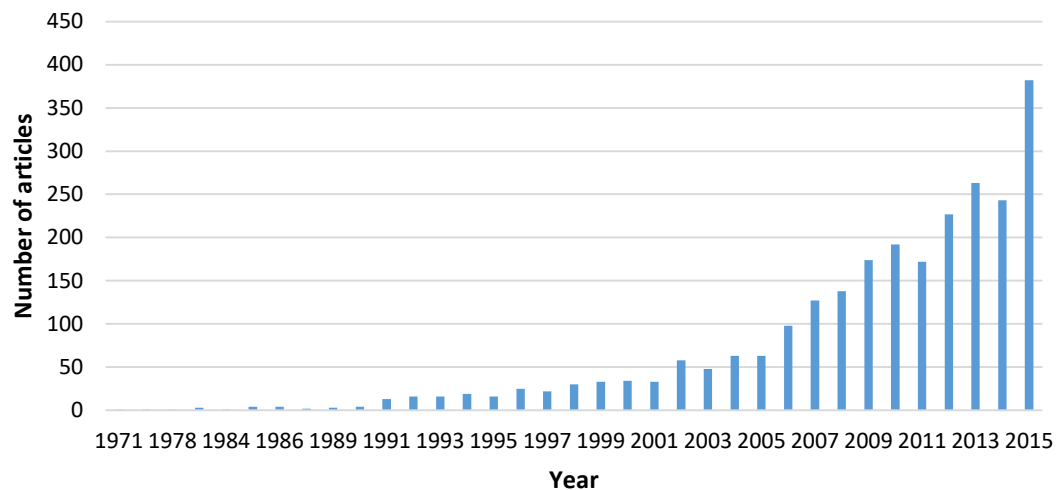


Figure 1-2. Increasing numbers of PD biomarker-related articles, as indexed by MEDLINE®, are appearing in the literature.

First, PD may be argued to be sufficiently prevalent in certain populations (e.g., patients with REM sleep behaviour disorder; Chahine et al., 2016) to justify screening (i.e., pre-pathological biomarkers). The disease has genetic forms as well as familial clustering of the idiopathic form, both of which allow for the use of pre-symptomatic biomarkers in, for example, twin-siblings of affected patients (Piccini, Burn, Ceravolo, Maraganore, & Brooks, 1999). Furthermore, because the neuronal loss in PD extends beyond the dopaminergic system, patients almost always develop non-motor symptoms, some of which predate the classical motor symptoms of PD (Chaudhuri & Naidu, 2008). This often persuades patients to seek medical attention, at which point an astute physician may also elect to use pre-clinical biomarkers to uncover an early PD diagnosis.

If the patient eventually displays ‘parkinsonian’ features, the treating doctor, especially if not a movement disorders specialist, may be faced with the conundrum of trying to confirm if the underlying pathology is indeed due to PD and not another cause of parkinsonism (e.g., multiple system atrophy). In such situations, a diagnostic biomarker would prove invaluable. Once PD is confirmed, clinical progression (e.g., cognitive impairment) and response to therapy may be monitored by progress and

monitoring biomarkers. Aware of their decline, patients may be interested in their prognosis (i.e., end-of-life biomarkers), and what they can be offered if end-stage disease develops (e.g., end-of-life biomarkers). Table 1-1 illustrates the multitude of examples of biomarkers currently or previously used in PD.

1.2.3.2 Challenges facing PD biomarker research

As with other neurodegenerative disorders, several hurdles face biomarker discovery in PD. Such challenges include the general complexity of the human CNS, limited access to and availability of tissue for histological diagnosis during the patient's lifetime and the relative lack of validated techniques for disease diagnosis (Dunckley, Coon, & Stephan, 2005). Added to these general obstacles are the diagnostic uncertainties surrounding PD, including disease heterogeneity and the potential for atypical parkinsonian syndromes.

As alluded to earlier, the multiplicity of disease aspects of PD make it difficult for a single multi-purpose biomarker to ever exist. This is evident from the current lack of such a 'Holy Grail' biomarker to date. By the same token, "reductionist"-type biomarkers focussing on single aspects of PD molecular neuropathology are unlikely to be overly clinically useful in the general schema of disease (Mielke & Maetzler, 2014). It seems, therefore, that several biomarkers of different types may need to be utilised in conjunction.

Taking PD diagnosis as an example, no one single biomarker to date has demonstrated perfect sensitivity and specificity; even post-mortem pathological examinations can be inconclusive at times (Berg et al., 2013; Lees et al., 2009). Instead, a tier-based system of different types of biomarkers (e.g., clinical assessment by a specialist, biofluid analysis, genetic testing and/or imaging studies) used in concert is

much more likely to yield the much needed diagnostic accuracy (Schlossmacher & Mollenhauer, 2010; Streffer et al., 2012).

Table 1-1. Examples of different PD biomarkers most of which had only limited or modest success.

Category Type	Pre-pathological	Pre-symptomatic	Pre-clinical	Diagnostic	Progress and monitoring
Bedside history and examination	Family history of PD (Barrett, Hac, Yan, Harrison, & Wooten, 2015)	Olfactory dysfunction (Braak et al., 2002)	Feelings of self- reproach (Huber, Freidenberg, Paulson, Shuttleworth, & Christy, 1990)	Cardinal triad of PD: bradykinesia with rigidity and/or tremor (Postuma et al., 2015)	MDS-UPDRS (Goetz et al., 2007)
Bedside tests	N/A	Subtle executive dysfunction (J. A. Cooper, Sagar, Jordan, Harvey, & Sullivan, 1991)	Abnormal colour discrimination (Buttner et al., 1995)	Surface EMG (Valls-Sole & Valldeoriola, 2002)	MoCA (Dalrymple- Alford et al., 2010)
Biochemical tests	Genetic analyses (Polymeropoulos et al., 1997)		Platelet CoQ ₁₀ (Gotz et al., 2000)	CSF and serum 8- hydroxyguanosine (Abe, Isobe, Murata, Sato, & Tohgi, 2003)	CSF A β ₁₋₄₂ (Siderowf et al., 2010)
Imaging studies	N/A	Dopaminergic deficits on PET (Laihinen et al., 2000)	Reduced dopamine transporter binding on [I-123]IPT SPECT (Schwarz et al., 2000)	Diminished putamen uptake on ¹⁸ F-dopa PET (Burn, Sawle, & Brooks, 1994)	¹⁸ F-dopa PET (Whone et al., 2003)
A β ₁₋₄₂ = amyloid β ₁₋₄₂ ; CSF = cerebrospinal fluid; CoQ ₁₀ = coenzyme Q ₁₀ ; [I-123]IPT = cocaine analogue; MDS-UPDRS = Movement Disorders Society-Unified Parkinson's Disease Rating Scale; MoCA = Montreal cognitive assessment; N/A = not available; PD = Parkinson's disease; PET = positron emission tomography; SPECT = single photon emission computed tomography.					

1.2.3.3 Progress and monitoring biomarkers

Of particular relevance to my biological markers study were progress and monitoring biomarkers. Given the protracted prodrome of PD, significant efforts have understandably been put into researching the potential use of biomarkers before the PD diagnosis is made (i.e., pre-pathological, pre-symptomatic and pre-clinical). Progress and monitoring biomarkers, on the other hand, have not until recently enjoyed as much attention (Michell et al., 2004). The importance of such biomarkers, however, cannot be underestimated.

First, the effect size of current PD treatments is rather arduous to quantify objectively. This stems from two reasons: PD symptoms exhibit modest fluctuations over time making it difficult to gauge improvement using clinical assessment alone (Clarke & Guttman, 2002); also, most such assessments tend to be subjective. Existing quantitative measures, such as the MDS-UPDRS, focus on motor deficits (Goetz et al., 2007) rather than providing a more holistic picture of the disease status.

Second, even if symptomatic fluctuation was discounted, relief of symptoms does not necessarily equate to a halted pathological process. In other words, PD symptoms do not make ideal surrogate biomarkers. For example, dopamine agonists, which possess proven neuroprotective properties (Nutt, Carter, Lea, & Sexton, 2002), also provide symptomatic relief. This symptomatic relief, in turn, confounds assessment of their underlying neuroprotective action. A better monitoring biomarker ought to detect retarded pathological progression independent of the patient's symptomatology.

Finally, appropriate progress biomarkers are needed to monitor the gradually deteriorating clinical course experienced by most PD patients. Whilst motor symptoms worsen over the course of the disease, it is the non-motor symptoms that often take the heaviest toll on the patient's quality of life (Schrag, Jahanshahi, &

Quinn, 2000). Cognitive impairment, in particular, places the greatest strain on PD patients (see review by Aarsland, Beyer, & Kurz, 2008), their caregivers and the wider society (Vossius, Larsen, Janvin, & Aarsland, 2011). In fact, even in non-PD patients, fear of dementia has far surpassed that of fear of any other disease (including cancer) and death (Benbow & Jolley, 2012). Therefore, there is an important need for a suitable progress biomarker that would serve as a surrogate end-point in future studies concerning PD-related dementia.

1.2.3.4 Future directions

Much research has gone into obtaining new prospectively-collected data (e.g., the Parkinson Progression Marker Initiative; Marek & PPMI Collaborators, 2011), as well as examining archived biofluid and tissue samples (e.g., Honolulu Asian Aging Study; Abbott et al., 2016). These efforts intend to make large datasets and standardised resources publicly available. The next major step, however, is properly utilising such data mines. This would manifest as using current bioinformatics and technological advances (i.e., “-omics”) to thoroughly evaluate the data, as well as making use of integration models which can capture the interplay between biomarkers that would have otherwise been hidden (Azuaje, 2011).

1.3 Blackcurrant anthocyanin trial

1.3.1 PD aetiology and pathogenesis

1.3.1.1 Background

The pathogenesis of neurodegenerative diseases, such as PD, has not been fully understood as yet. It appears to be complex and multifactorial; and to encompass

both genetic and non-genetic causes. Since sporadic (i.e., non-genetic) cases of PD are the majority, this section will examine some of the proposed mechanisms of the pathogenesis of PD that have been reviewed in the medical literature.

1.3.1.2 RNA damage, protein misfolding and lipid peroxidation

A growing body of evidence is now indicating that PD perhaps arises from reactive oxygen species (ROS)-induced dysfunction of subcellular structures. Shan *et al* have shown that oxidation of ribonucleic acid (RNA) is an incident occurring prior to neuronal cell death (2007). This damaged RNA leads to a decline in protein expression and is associated with neural deterioration seen in neurodegenerative disease, such as AD and PD. Likewise, oxidative damage to RNA can result in misfolding of proteins, initiating a cascade of abnormal protein production. In PD, this protein is α -synuclein, and has been shown to aggregate to form Lewy bodies—which were found to be toxic to neurons (Spillantini et al., 1997). Lastly, lipid peroxidation was reported to account for some of the neural changes seen in neurodegenerative disorders. Lipid peroxidation results from damage to neural cell membranes induced by ROS. Cell membranes become more susceptible to ROS-induced damages as a result of a decrease in fluidity of these membranes due to increased dietary lipid consumption as well as aging (Tacconi, Lligona, Salmona, Pitsikas, & Algeri, 1991). RNA damage, protein misfolding and lipid peroxidation are in part due to ROS and the oxidative stress (OS) experienced by neuronal cells, which is the subject of the following section in the chapter.

1.3.1.3 Disruption of cerebral microcirculation

In a study led by Farkas *et al*, it was found that there was a severe disruption of limbic cingulate cortex microvasculature in patients with PD and AD (2000). The cingulate gyrus is a very important region of the brain in the study of neurodegenerative

disease pathogenesis as it displays characteristic pathological features, such as neurofibrillary tangles and Lewy bodies, seen in patients with AD and PD, respectively. The authors of the study attributed the microanatomical disruption of the vessels (e.g., capillary basement membrane thickening) to an alteration of the internal metabolism of the endothelium. This metabolic change was due to the toxic influence that ROS had on the production of basement membrane constituents of these microvessels.

1.3.1.4 Decreased cerebral perfusion

Another suggested cause in the aetiology of PD is decreased blood flow to certain parts of the brain (Kawabata, Tachibana, & Sugita, 1991). Kawabata *et al* (Kawabata et al., 1991) found subjects with PD to have decreased blood flow to frontal and temporal cortices, basal ganglia and thalamus when compared with controls. This decline in regional blood flow can be caused by disruption of blood vessels as described earlier, but no study has systematically examined this relationship.

1.3.1.5 Substantia nigral susceptibility

Susceptibility of the substantia nigra to excessive neuroinflammation and exposure to ROS, both leading to neurodegeneration, has been implicated in the pathogenesis of PD. One study has shown that the substantia nigra contained four to eight times higher the number of microglial cells (i.e., part of the immune system) compared with the hippocampus and cerebral cortex (Kim et al., 2000). Activated microglial cells secrete defence substances, such as tumour necrosis factor-alpha and superoxide, which can be toxic to neurons of the substantia nigra. Hence, the substantia nigra is at an added risk of immunologic insults compared with the rest of the brain. This has been verified by Liu *et al* who treated cultures of mesencephalic neuroglia with dextrorotatory morphine (i.e., an over-the-counter antitussive drug), and found that it

hindered the activation of microglia and thereby protected substantia nigral cells (2003). Moreover, exposure to ROS produced during dopamine metabolism in dopaminergic cells further increase the vulnerability of the substantia nigra to undergo degeneration (Lloyd, 1995).

1.3.1.6 Excitotoxicity

Excitotoxicity refers to a state of neural cell death due to over-excitation of these neurons by an excitatory neurotransmitter. Glutamate is the brain's major excitatory neurotransmitter, and its receptors are especially abundant in dopaminergic cells of the substantia nigra (Yacoubian & Standaert, 2009). The mechanism by which over-excitation of neurons results in cell death is not clearly understood. One mechanism reported by Rothman suggested that glutamate causes steady depolarisation of neurons, which leads to an influx of chloride ions into the neurons (1985). As a result, cations and water are attracted by electric and osmotic gradients into the neurons, causing cell lysis. Another proposed mechanism connects neural cell death to an excessive influx of calcium ions into neurons as a result of glutamate stimulation (Choi, 1988). This pathological increase in intracellular calcium concentrations leads to many detrimental effects on the cell, including neurofilament proteolysis and mitochondrial dysfunction (Gilbert & Newby, 1975). Figure 2-3 illustrates the proposed pathway of these mechanisms.

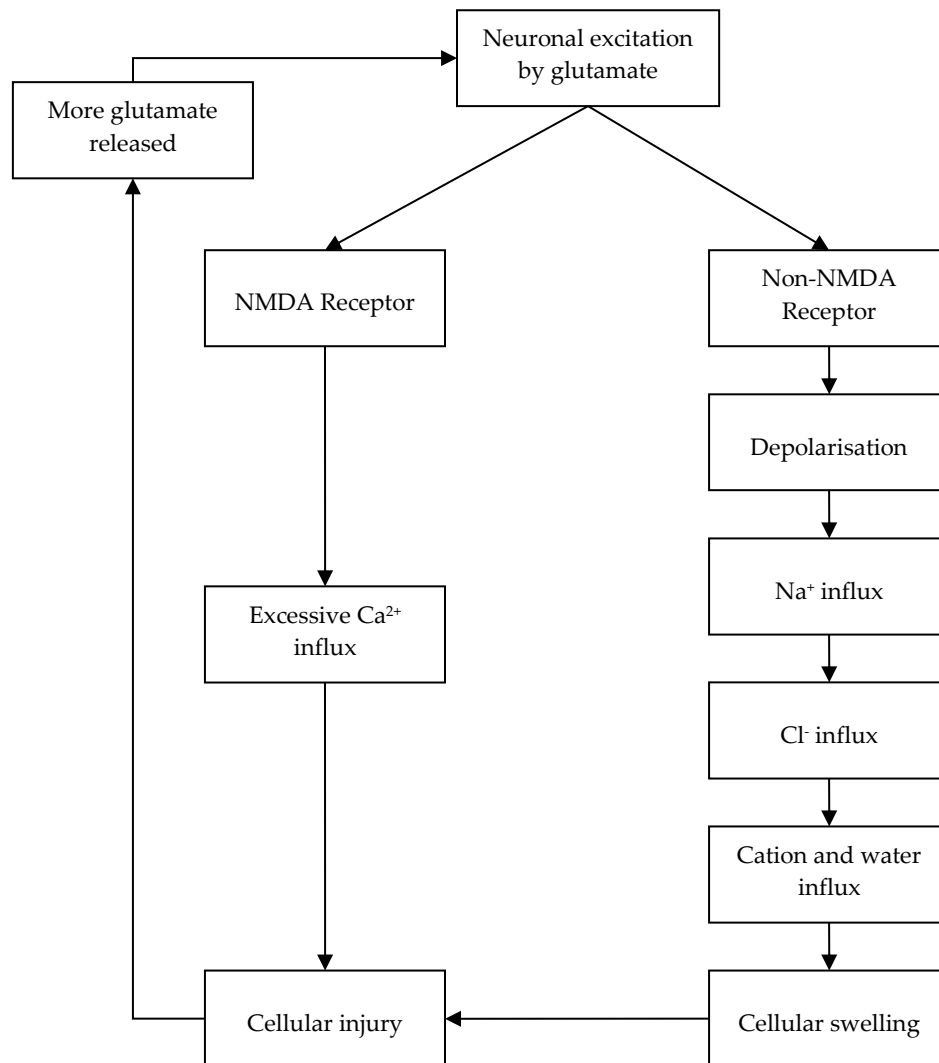


Figure 2-3. The cascade of events leading to neuronal cell death due to glutamate-induced excitotoxicity (adapted with permission from Choi, 1988); Ca²⁺ = calcium; Cl⁻ = chloride; NMDA = N-methyl-D-aspartate; Na⁺ = sodium.

1.3.2 Oxidative stress

1.3.2.1 Background on free radicals

A free radical is any chemical species possessing one or more free electrons. To reach a more stable state, free radicals act as electron acceptors from other molecules, and are, hence, more reactive compared with the non-radical form of the atom or molecule (Gilgun-Sherki, Melamed, & Offen, 2001). Examples of intracellular free

radicals include superoxide and hydroxyl species. Other related chemical species include hydrogen peroxide and peroxynitrite, and—although not free radicals themselves—they substantially influence cellular redox state. These molecules are collectively known as ROS or as prooxidant species (Simonian & Coyle, 1996).

There are three major sources of ROS generation in neurons: mitochondrial oxidation metabolism, enzymatic reactions involving mixed-function oxidation, and autoxidation of small molecules. Accumulation of such chemical species can result in neural dysfunction or even death (Simonian & Coyle, 1996). However, the body has an endogenous mechanism of defence via the antioxidant defence mechanisms. Antioxidant defence mechanisms function through removal of oxygen, scavenging of ROS or their precursors, inhibition of ROS formation, binding of metal ions needed for ROS formation and upregulation of intracellular antioxidant defences (Gilgun-Sherki et al., 2001). The antioxidant system can be categorised into two groups: enzymes, such as superoxide dismutase and glutathione peroxidase, and low molecular weight antioxidants, such as glutathione, tocopheroles (i.e., vitamin E) and lipoic acid.

1.3.2.2 Oxidative stress

Oxidative stress (OS) is the cytopathological consequence of an imbalance towards the prooxidant side in the prooxidant/antioxidant homeostasis (Zhao, 2009). It is the result of an increase in ROS production, a decrease in antioxidant defence mechanisms or both. The CNS is one of the body systems most exposed to OS (M. A. Smith et al., 1998; M. A. Smith et al., 1996). It is particularly vulnerable due to high metabolic rate and high rate of oxygen consumption, long exposure to neuroinflammation via activated microglia, reduced capacity of neural regeneration (i.e., post-mitotic cells), higher contents of polyunsaturated fatty acids, and relatively

low levels of endogenous antioxidant defences (Andersen, 2004; Perry, Godin, & Hansen, 1982; Tacconi et al., 1991).

Oxidative stress has been implicated as a possible initiating event in the aetiology to neurodegenerative diseases. It is not, however, clear whether OS is a cause, an epiphenomenon or a consequence of neurodegeneration (Papandreou et al., 2009). One hypothesis of a causal relationship — proposed by Jenner and Olanow — takes into account a combination of two experimental findings (1996). Firstly, oxidation of the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is known to be selectively toxic to dopamine terminals in the striatum, to its metabolites 1-methyl-4-phenylpyridinium (MPP⁺) and 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) has been shown to cause mitochondrial dysfunction, a state of OS and generation of ROS in dopaminergic cells (Chiba, Trevor, & Castagnoli, 1984; Johannessen, Adams, Schuller, Bacon, & Markey, 1986). Secondly, autooxidation of dopamine has also been demonstrated to generate ROS and to oxidatively stress dopaminergic cells of the substantia nigra. Although a causal relationship between OS and neurodegenerative diseases is thus feasible, the true cascade of events remains uncertain. For example, Andersen has recently suggested that OS might only be a part of a ‘cycle’ of insults leading to neurodegeneration, rather than a ‘cascade’ of events (2004). Refer to Figure 2-4 below for an illustration of Andersen’s proposed model of the role of OS in the aetiology of neurodegenerative diseases.

1.3.3 Neuroprotection

1.3.3.1 Rationale for using neuroprotective agents in PD

Neuroprotective agents in PD are defined by Yacoubian and Standaert as “therapies that slow or prevent further neurodegeneration of neural populations involved in PD, both dopaminergic and non-dopaminergic”. Thus, they exclude ‘neurorestorative’

strategies, such as stem-cell transplantation, that aim to replace neuronal cells after they have been lost (2009). Because the course of neuronal loss in neurodegenerative disorders in general — and in PD specifically — is years-long and slow, it is of clinical significance to utilise this time-lag to try to delay or prevent further neurodegeneration by the use of neuroprotectants. Slowing the progression of PD has always been an unmet medical need for clinicians and patients alike, and is now a major focus of research (Kiebertz, 2006). If slowing or halting neurodegeneration could be achieved, this would delay the decline in motor symptoms, preserve patients' quality of life, and perhaps avoid the incapacitating complications of advanced PD (Suchowersky et al., 2006; Yacoubian & Standaert, 2009).

An ideal neuroprotectant has been described in the medical literature to possess certain characteristics. For instance, it should be able to interact with molecules in both hydrophilic and lipophilic environments to ensure maximal protection of cells. It also has to have minimal cell toxicity, which widens the range of concentration within which it can exert neuroprotective effect and improve cell survival. In addition, it should preferably be attainable from 'natural' sources, such as dietary herbals and nutritional supplements (Chao, Yu, Ho, Wang, & Chang, 2008; Sies, 1997). Such 'natural' sources can be better perceived by patients than 'chemical' drugs, which can increase adherence to taking medications (Jin, Sklar, Min Sen Oh, & Chuen Li, 2008). This is pertinent because several studies have shown that PD patients commonly use complementary and alternative therapies in addition to their prescribed medications. Two studies from the United States (US) reported that 40 to 63% of PD patients use at least one nutritional supplement or alternative therapy (Rajendran, Thompson, & Reich, 2001; Wolfrath et al., 2006). Another study from the United Kingdom has shown that up to 44% of PD patients used complementary therapies (Ferry, Johnson, & Wallis, 2002). The most common non-prescribed medications and therapies included vitamin supplements, herbs, cod liver oil, massage, acupuncture and aromatherapy (Ferry et al., 2002).

One of the most important characteristics of a putative neuroprotective agent for PD is that it should directly target factors in the pathogenesis of the disease. Since the pathogenesis of PD is now thought to be multifactorial (as reviewed in previous sections), it would be advantageous to use neuroprotectants that can target multiple mechanisms of aetiology at once. Therefore, using Andersen's model of PD pathogenesis (Figure 2-4), it can be deduced that a therapy targeting ROS formation can have a multiplicity of benefits. One such class of neuroprotectants is antioxidants.

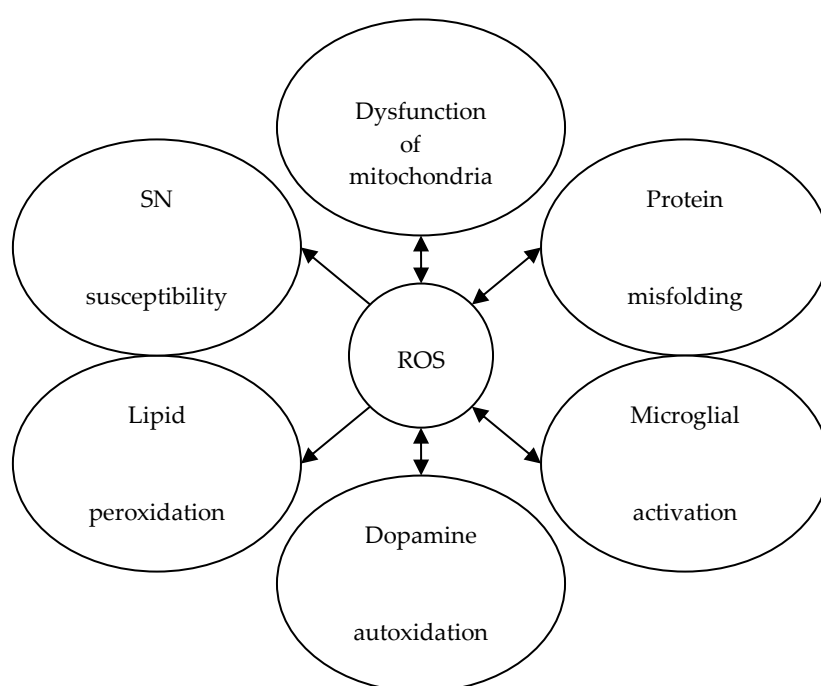


Figure 2-4. Reactive oxygen species appear to be central to the aetiological processes in PD, either by directly causing damage (one-way arrow) or inciting a vicious cycle of damage (two-way arrow); ROS = reactive oxygen species; SN = substantia nigra (reproduced with permission from Y. Alamri, 2015).

An antioxidant can be defined as any chemical molecule that when present in lower concentrations than the substrate to be oxidised, can retard or avert the autoxidation or ROS-mediated oxidation of the substrate, resulting in a more stable substrate (Halliwell, 1989; Salah et al., 1995). In addition, Salah *et al* have shown that the more hydroxide groups present in a molecule, the greater its antioxidant activity (1995). Classification of antioxidant systems has already been alluded to the previous section. Antioxidants can be soluble in water (i.e., hydrophilic) or in lipids (i.e., lipophilic).

Furthermore, they can be synthesised by the cells (e.g., glutathione) or obtained from the diet (e.g., polyphenols found in plant-based foods; Sies, 1997). Antioxidants have been shown to protect CNS against OS in *in vivo* and *in vitro* studies. Although their exact mechanism of action is still unclear, a number of mechanisms have been proposed including: direct scavenging of ROS and inhibition of neuronal apoptosis through inhibition of certain intracellular enzymes (Schroeter, Spencer, Rice-Evans, & Williams, 2001).

1.3.3.2 Previous trials of neuroprotective agents in PD patients

Clinical trials using neuroprotective agents in patients with PD have had disappointing results. This section aims to highlight some trials of antioxidant agents tested as a neuroprotectant and their results.

Coenzyme Q₁₀

Coenzyme Q₁₀ (CoQ10), also known as ubiquinone, is an electron acceptor for mitochondrial complexes I and II/III, and thus acts as an essential cofactor in the electron transport chain. CoQ10 has been shown to be a potent antioxidant as it slowed the loss of striatal dopaminergic cells in animal studies (Beal, Matthews, Tieleman, & Shults, 1998). Trials of its use in human subjects have yielded inconsistent data, however. Two large randomised trials have failed to show a statistically significant benefit of CoQ10 in patients with PD, and deemed it not different to placebo (NINDS Net-PD Investigators, 2007; Storch et al., 2007).

Mitoquinone

Mitoquinone (MitoQ) is an orally active mimic of CoQ10, but with a substantially higher antioxidant capacity. It is the first drug developed of its class. Although it has shown encouraging results in *in vivo* studies, proof of benefit in human subjects with

PD is still lacking (Tauskela, 2007). Phase II clinical trials are still ongoing at this stage.

N-acetylcysteine amide

N-Acetylcysteine amide is a novel low molecular weight thiol antioxidant that has been developed by Bahat-Stroomza *et al* (2005). N-Acetylcysteine has been found to cross the blood-brain barrier (BBB) in a rat model. Furthermore, N-acetylcysteine amide was capable of slowing the rate of dopaminergic cell loss and dopamine level decline using three different PD models (Bahat-Stroomza et al., 2005). The efficacy of N-acetylcysteine amide has not been demonstrated in humans yet.

Dietary vitamins

Consumption of foods rich in vitamins, such as vitamin A, C and E, has long been associated with a lower incidence of PD, as shown in a number of population-based studies (de Rijk et al., 1997; Fahn, 1991; Logroscino et al., 1996). However, in clinical trials, dietary vitamin supplementation was shown not to conduct any benefit against developing PD. For example, investigators of the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trial came to the conclusion that vitamin E was completely ineffective at slowing the decline of PD patients who were in the early stages of the disease (Shoulson, 1989).

1.3.3.3 Why have neuroprotective agents failed so far?

As promising as the concept of neuroprotection is, development and clinical use of such agents have been slow and of limited success. This stems from many factors which can largely be grouped into three categories: limited knowledge, methodological challenges in pre-clinical studies and methodological challenges in clinical studies. This section will discuss each category in more detail.

Limited knowledge

Due to the complexity of PD pathology, and the interplay of several environmental and genetic factors associated with its pathogenesis, it is difficult to define PD as a single entity with one primary cause. Rather, PD should be viewed as a syndrome encompassing multiple disorders with different first presentations and mechanisms of aetiology (Leventoglu & Baysal, 2008; Yacoubian & Standaert, 2009). It can be difficult to prove definite neuroprotective effects of an agent in a group of patients with such a heterogeneous disease. For example, different 'types' of PD could be due to different combinations of genes, each of which responds differently to a certain neuroprotective therapy. Furthermore, it is important to determine at which stage of the disease (i.e., early, established or advanced PD) neuroprotective therapies should be started. For example, a number of studies have suggested that neuroprotective therapies should be used as prophylactics for high-risk groups, such as the elderly. This is because neuroprotectants may not, in fact, be able to protect cells that have been irreversibly damaged by prolonged exposure to toxic environments (Bahat-Stroomza et al., 2005; Gilgun-Sherki et al., 2001; Hung & Schwarzschild, 2007). Finally, there is insufficient knowledge as to whether antioxidants actually cross the human BBB when administered systemically, and this warrants more studies on the molecular and cellular transport processes of the brain capillary endothelial system (Gilgun-Sherki et al., 2001).

Methodological challenges in pre-clinical studies

Methodological challenges in the setting of pre-clinical studies can either be due to the choice of the neuroprotectant itself, or to the methods used to analyse the neuroprotective effects it exerts. Identification of a suitable candidate neuroprotectant is very important, as is adequate purity of the sample and the optimal dose to be used. Inadequacies in such factors can result in mixed or even negative results (Gilgun-Sherki et al., 2001; Hung & Schwarzschild, 2007). Moreover, appropriate evaluation of the neuroprotective potential of a candidate agent is essential to

produce consistent results. For instance, some studies solely rely on radical scavenging tests as a primary indicator of the antioxidant activity of an agent (Kähkönen, Heinämäki, Ollilainen, & Heinonen, 2003). While radical scavenging potential is an important parameter to consider, there are many other factors, such as interaction with metal ions and other antioxidants, that also determine the antioxidant activity of an agent, which should also be examined.

Methodological challenges in clinical trials

One of the most frequent difficulties encountered in clinical studies is how to define 'neuroprotection' in a context where no direct tests are readily available. For example, no direct visualisation of neurons in the substantia nigra is possible during the patient's life (Kieburtz, 2006). Therefore, in order to determine the rate of neuronal loss as a way of monitoring neuroprotective activity, a surrogate marker needs to be employed. Many PD surrogates have been proposed in the medical literature, but they are far from optimal. For example, clinical surrogates, such as time delay to the requirement for levodopa, have not been fully validated yet (Ravina et al., 2005). Similarly, imaging surrogates, such as ^{18}F fluorodopa positron emission tomography, were shown to be confounded by the therapeutic effects of the neuroprotective intervention (Clarke & Guttman, 2002; Kieburtz, 2006). Finally, since neural degeneration of PD occurs slowly and gradually, studies ought to be evaluating the patients over a long period of time (Kieburtz, 2006).

1.3.4 Blood-brain barrier

1.3.4.1 Overview of the Brain-barrier system

The brain is one of the most active organs in the body, receiving about 15% of total cardiac output and utilising around 20% of the body's oxygen and glucose (Saladin, 2010). Despite this large demand of blood, the brain needs to protect itself from

potential blood-borne harmful agents, such as antibodies and macrophages. Furthermore, it requires a tightly-regulated environment, which is achieved by selectively transporting some molecules, but not others, between the blood and the brain's extracellular fluid (ECF). These molecules may either pass in their original form, or require some sort of metabolism/modification before transportation (Risau & Wolburg, 1990). A barrier system (known as the brain-barrier system 'BBS') helps achieve these requirements whilst fitting into the microenvironment between the brain tissue and blood vessels.

There are potentially two points of entry that need to be protected: the capillaries of the choroid plexuses and the capillaries throughout the brain tissue. Still however, there remain regions of the brain that are fully exposed to blood. These are close to the third and fourth ventricles, and are hence termed circumventricular organs (CVOs; Risau & Wolburg, 1990). This access to blood is important for free exchange of molecules between brain ECF and blood, and to allow the brain to monitor and respond to fluctuations in blood potential of hydrogen (pH), glucose, osmolarity, neuroendocrine hormones and other variables. However, CVOs also offer a potential route to pathogens, such as human immunodeficiency virus, to enter and invade the brain.

The blood-CSF barrier (BCB) protects the brain at the choroid plexuses and is made up of ependymal cells joined by complex tight junctions (Saladin, 2010). Ependymal cells elsewhere in the brain, however, lack tight junctions, since it is important to allow for exchange between CSF and brain ECF (i.e., there is no brain-CSF barrier). Tight junctions prevent free passage of molecules between blood and CSF, but a number of factors, such as chronic alcohol intake, hypertension and high body weight, can cause a disruption of BCB. This leads to a 'leakage' of protein into CSF, measured as CSF/serum albumin ratio (Kornhuber, Kaiserauer, Kornhuber, & Kornhuber, 1987). The disruption of BCB has also been implicated in the pathogenesis

of neurodegenerative diseases. This relationship is not fully understood however, as Haussermann *et al* have shown that BCB was actually intact in patients with early PD (2001).

The BBB is an endothelial protective layer present in the capillaries coursing the brain tissue (Youdim et al., 2003). This endothelial barrier has a number of characteristics that make it different from any other endothelial barrier in the body. Firstly, cerebral endothelial cells have complex tight junctions that are narrower than other tight junctions, and form an 'unbroken belt' between the cells, unlike anywhere else in the body. This barrier prevents transportation of molecules through the paracellular route (Reese & Karnovsky, 1967). Secondly, cerebral endothelial cells have fewer pinocytotic vesicles (i.e., a class of vesicles that allows the cell to take up molecules from the surrounding milieu) compared with other endothelial cells, and hence limit the passage of molecules transcellularly (Reese & Karnovsky, 1967). Finally, BBB cells possess certain uptake and efflux transport systems and metabolic enzymes that help regulate brain ECF (Youdim et al., 2003).

Two mechanisms have been proposed whereby endothelial cells differentiate to become a BBB: predetermined neuroectoderm-derived angioblasts differentiating into BBB endothelium, or endothelial cells from outside CNS invading neuroectoderm and responding to the neural environment (i.e., astrocyte stimulation) by differentiating into BBB (Risau & Wolburg, 1990; Saladin, 2010). The latter proposal has garnered more support from published studies including stem-cell transplantation experiments (Risau & Wolburg, 1990). Moreover, Qin and Sato have found the first appearance of morphologically-distinct endothelial cells in neural tissues during BBB development in mouse-embryos to be on embryonic day 10.5 (1995). However, the timing of BBS formation in humans remains uncertain as it is much more difficult to assess BBS function in human embryos (Saunders, Dziegielewska, & Møllgaard, 1991).

1.3.4.2 Mechanisms of crossing the BBB

In order for a therapeutic agent to be able to exert its effects on a targeted tissue or organ, it needs to be able to access it. For neuroprotective therapies, for instance, this translates into the requirement of crossing from the blood into the brain through the BBS. There are a number of mechanisms that various molecules use to cross the BBS, the most important of which will be described in this section. Since the blood/brain interface is more important in terms of the clinical use of neuroprotectants, only the BBB will be considered in this section, although similar principles of molecule passage apply equally to the BCB.

Cellular permeability through the BBB

Normally, only very small quantities of hydrophilic molecules are able to diffuse through tight junctions of cerebral endothelial cells (i.e., paracellular pathway). Lipophilic molecules, on the other hand, utilise the transcellular pathway—a faster and more efficient route for molecular trafficking. Such pathways can be exploited as a method of drug delivery across the BBB. For example, addition of a lipophilic group or a lipophilic transport vector, such as a nicotinoyl residue, to a drug can enable it cross the BBB and get into the brain (Gilgun-Sherki et al., 2001). In addition, since essential molecules (e.g., glucose and nucleotides) have specific membrane-bound transporters which are present at high concentrations in cerebral endothelial cells, drugs whose molecules closely resemble essential molecules may also be transported through the same transporters. Finally, macromolecules can occasionally be shuttled into the brain via receptor-mediated and adsorptive endocytotic systems; and this can be utilised for drug delivery as well (Gilgun-Sherki et al., 2001).

Physiological opening of the BBB

Since not all drugs can be easily modified to become more permeable to the BBB, researchers have been trying to develop other techniques that enable a transient

opening of cerebral endothelial cells' tight junctions. Greenwood has shown that inflammatory mediators of the body induced a transient opening of the BBB (1992). Not only is this finding clinically important, but it also forms the basis of a potential way to open the BBB transiently, but that is well-tolerated by the brain.

Induced opening of the BBB

Induced opening of the BBB can have some advantages over physiological opening in that it can offer a more controlled and selective opening of the BBB. It can also be more precisely timed if, for example, an agent can be given to induce opening of the BBB and then another to close it. Osmotic opening refers to opening of the BBB induced by the use of a hypertonic solution, such as mannitol, which is normally administered via an intracarotid injection. Mannitol causes cerebral endothelial cells to shrink, opening tight junctions for a window of few hours. This technique has been used to deliver chemotherapeutic agents to patients with brain tumours (Gilgun-Sherki et al., 2001). Chemical opening, on the other hand, employs the use of isotonic pharmacological molecules to cause the BBB to open. For example, Black *et al* have shown that leucotriene C₄ causes a temporary opening of the BBB (1990).

Pathological opening of the BBB

Disruption of the BBB can be a complication of major head trauma. However, other pathological processes have also been linked to more subtle disruption of BBB and capillary permeability. These pathological processes include cerebral ischaemia and multiple sclerosis ('MS'; Friedman et al., 1996; Gilgun-Sherki et al., 2001; Harris, Frank, Patronas, McFarlin, & McFarland, 1991).

1.3.4.3 Clinical assessment of whether molecules cross the BBB

If a neuroprotective agent is to be developed and given to patients with PD, it first has to pass through the BBS and get into the brain. However, it is not an easy task to

directly detect if these molecules do pass into the brain, as there are no simple laboratory tests available to do that. If a neuroprotectant does cross the BBS however, then it should also be present in the CSF, since there is no brain-CSF barrier. CSF can be clinically tested in the laboratory to determine whether certain molecules are present in the CSF or not. Samples of CSF can be obtained through a lumbar puncture (LP). An LP is a common procedure that is used to gain access to the fluid-filled space in the meninges in order to withdraw samples of CSF, measure CSF pressure or administer drugs or anaesthesia (N. Cooper, 2002). Although an invasive procedure, it carries less risk than, for example, having to biopsy the brain to show that molecules of the neuroprotective agent are present.

Due to LP's invasive nature and its other associated complications (e.g., headache), it is not practical to use it as a way of routinely administering the neuroprotectant, especially if multiple doses are required. On the other hand, studies have shown that when antioxidants were added to animals' diets, researchers were able to detect antioxidant metabolites in these animals' brains (Kalt et al., 2008). Therefore, dietary antioxidants were able to cross the BBS in these animals; and if these results could be replicated in human subjects, it would be of potential therapeutic interest to PD and other neurodegenerative disease patients. The next section will describe a sub-class of dietary antioxidants, anthocyanins, which have shown some promise in pre-clinical studies.

1.3.5 Blackcurrant anthocyanins

1.3.5.1 Anthocyanins: Introduction and dietary sources

Anthocyanins are a group of over 500 water-soluble pigments that give a plant its red, blue or purple colours. They are often referred to as 'secondary compounds'; compounds that do not usually contribute to the plant's primary metabolism. Rather,

they serve ecological functions that enhance the plant's survivability—for example, anthocyanin pigments have been shown to attract pollinators and seed dispersers (Winkel-Shirley, 2001). Anthocyanins are thought to be involved in the health-related benefits of consuming fruit and vegetables (Andres-Lacueva et al., 2005). The average adult intake of anthocyanins in European and US diets is estimated to be around 180 to 250 mg per day (Kuhnau, 1976). However, this was reported in individuals consuming several servings of fruit and vegetables, and 'usual' intake is probably much less (Hertog, Hollman, Katan, & Kromhout, 1993; Kuhnau, 1976; Prior, 2003).

Anthocyanins belong to a larger family of compounds called flavonoids. Flavonoids are plant polyphenolic compounds (i.e., contain more than one phenol unit); and are categorised (see Figure 2-5) into flavonols, flavones, flavanols and anthocyanins (Kuhnau, 1976). Anthocyanins are the most consumed flavonoids in European and US diets compared with other flavonoids (Hertog et al., 1993).

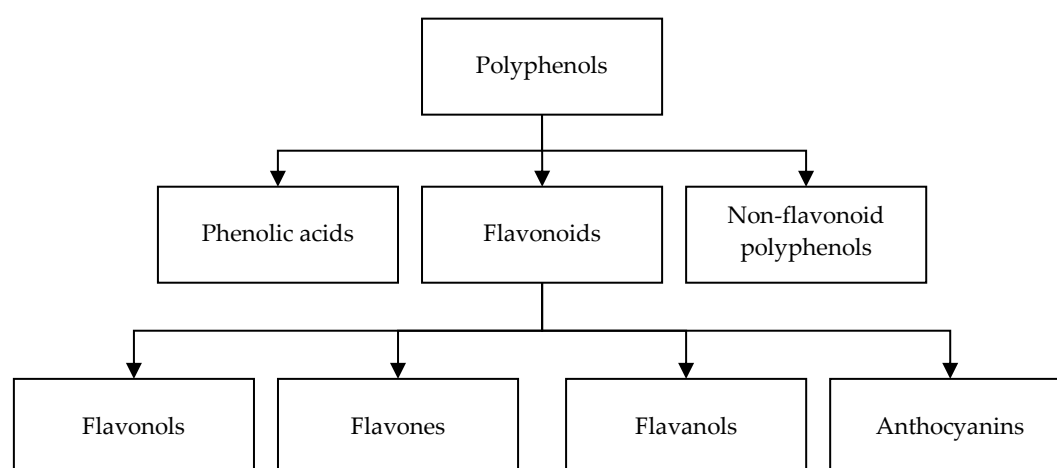


Figure 2-5. Classification of the polyphenolic compounds (adapted with permission from Weinreb, Mandel, Amit, & Youdim, 2004).

Almost all dietary anthocyanin intake comes from colourful fruit and vegetables. Kalt *et al* found that the brains of pigs fed on diets restricted to oats, barley and soy contained almost undetectable levels of anthocyanins (2008). Among edible plants, fruit and vegetables with deep red, blue or purple colours are among the richest

sources of anthocyanins. These include berries—such as grapes, blackcurrants and blueberries—red cabbage, red onions and eggplants (Wu et al., 2004). In addition to fresh fruit and vegetables, extracts from these plants, including juices, wines and jam, are also rich in anthocyanins (Kähkönen et al., 2003).

Anthocyanins have unique chemical properties. The colours of anthocyanins are affected by their chemical nature, concentration and the pH (Brouillard, 1983). They are very active compounds, and have been shown to be most stable in acidic environments (i.e., between pH 1.0 and 2.4; Cabrita, Fossen, & Andersen, 2000). Moreover, Walton *et al* have found that anthocyanins become less stable or even degrade when they are exposed to decreased acidity (i.e., pH beyond 4.5) or increased temperature (i.e., beyond 60 C; 2006). Similarly, the presence of oxygen or light, or exposure to infrared radiation, were also found to decrease anthocyanins' stability in *in vitro* studies (Xiong, Melton, Easteal, & Siew, 2006). Xiong *et al* documented that encapsulating anthocyanins with natural polymers, such as pullulan, preserved the stability of some of the anthocyanins; and that degradation products retained the antioxidant activity of anthocyanins (2006).

1.3.5.2 Anthocyanins as part of functional foods

Functional foods have been defined as “foods that are part of a normal diet which have been fortified or enriched to provide additional health promoting benefits in conjunction with normal nutritive properties” (Devcich, Pedersen, & Petrie, 2007). A wide range of literature has been published on the health benefits of consuming anthocyanin-rich fruit and vegetables. This section will review some of these reported health benefits from human and animal studies (see review by Y. Alamri, 2010).

Antioxidant activity

Anthocyanins have been the focus of many studies examining different dietary sources of antioxidants. In *in vitro* studies, anthocyanins were shown to protect against OS and deoxyribonucleic acid (DNA) damage when human cultured cells, such as human neuroblastoma cells and human promyelocytic cells, were incubated in anthocyanin-rich solutions (Ghosh, McGhie, Zhang, Adaim, & Skinner, 2006). Bub *et al* have found that DNA oxidative damage (i.e., oxidised DNA bases) was significantly reduced in healthy human subjects who were given polyphenol-rich juices for two weeks (2003). Similarly, Cho *et al* reported that consumption of purple sweet potato anthocyanin resulted in the inhibition of lipid peroxidation in brain extracts of rats (2003). However, results from other human and animal studies have been less promising and shown mixed results. For example, when a group of elderly patients were given polyphenol-rich desserts for two weeks, investigators found no change in participants' antioxidant status (Carmen Ramirez-Tortosa *et al.*, 2004).

Effects on immunity

Anthocyanins may also exert effects on the immunological status of people who consume anthocyanin-rich fruit and vegetables. When human subjects were given polyphenol-rich juices to consume, it was found that activated lymphocytes secreted more interleukin 2 and that it was easier to mount an immune reaction against pathogens in these subjects. In addition, natural killer lymphocytes had an enhanced 'killing' ability due to anthocyanin consumption (Bub *et al.*, 2003).

Memory enhancement

The use of anthocyanins has also been implicated in the prevention of cognitive and behavioural losses seen with aging. Hence, research has focused on trying to improve memory by the supplementation of anthocyanin-rich diets. Andres-Lacueva *et al* found that rats fed blueberry anthocyanins and anthocyanin metabolites performed

better in cognitive tests, such as the Morris water maze (2005). This indicates anthocyanins may have been present in the rats' brains. Another study also found effects of anthocyanins on improving cognitive performance in ethanol-treated rats (Al-Habeeb, 2003).

Effects on blood vessels

Anthocyanins also appear to have a favourable vasodilatory action on the vascular system. Nakamura *et al* have shown that bathing rat aortic ring in an anthocyanin-rich solution induced a dose-dependent vasorelaxation (2002). Furthermore, in a double-blind placebo-controlled human trial, subjects who consumed an anthocyanin-rich juice were found to have an increase in forearm blood flow (Matsumoto *et al.*, 2005). These favourable effects on blood vessels are hypothesised to be due to an anthocyanin-induced increase in nitric oxide synthesis, resulting in an endothelium-dependant vasorelaxation via type-1 histamine receptors (Y. Nakamura *et al.*, 2002).

Effects on hyperglycaemia and hyperlipidaemia

The consumption of anthocyanin-rich fruit and vegetables has been linked to a lower risk of developing diabetes mellitus and hyperlipidaemia (Tsuda, Horio, Uchida, Aoki, & Osawa, 2003). This might be attributed to the fact that consuming 'healthy' foods can mean less space for 'bad' foods. However, Tsuda *et al* found that dietary intake of anthocyanins helped improving insulin resistance precipitated by high fat diets in mice. Furthermore, they have found that anthocyanin-rich diets decreased levels of enzymes responsible for the synthesis of fatty acids and triacylglycerols (2003).

1.3.5.3 Why blackcurrants?

Blackcurrants (*Ribes nigrum* L.) are a species of berries that are commonly consumed as part of the diet in many parts of the world, including New Zealand. In fact, New Zealand is known for its mass production of high quality blackcurrants. Currently, Japan imports 3.6 million US dollars' worth of New Zealand blackcurrants to use in several products including snacks, supplements, jam and cosmetic products (Hansen, 2009). Although inedible, leaves and buds of blackcurrants are also used in the production of some food supplements (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006).

Blackcurrants are among the most anthocyanin-rich of berries. They contain around 250 mg of anthocyanins per 100 g of fresh fruit (Koeppen & Herrmann, 1977). Amakura *et al* reported that blackcurrants ranked second in their radical scavenging ability among nine other berries, behind blackberries (2000). Furthermore, Tabart *et al* have found that compared with blackcurrant berries, leaves and buds contained three times the concentration of phenolic components and six times the antioxidant activity (2006).

Another factor that favours the use the blackcurrants in research is their relatively simple composition; they contain four major anthocyanins, which account for about 97.2% of the anthocyanin content in blackcurrants (Slimestad & Solheim, 2002). First reported by Chandler and Harper (1962), these four anthocyanins are delphinidin 3-O- β -glucoside (D3G), delphinidin 3-O- β -rutoside (D3R), cyanidin 3-O- β -glucoside (C3G) and cyanidin 3-O- β -rutoside (C3R). This simple composition makes blackcurrants more convenient to assess for structure-activity relationships and bioavailability studies, compared with other berries, such as bilberries—which contain around 15 different anthocyanin components (Nakaishi, Matsumoto, Tominaga, & Hirayama, 2000).

Therefore, blackcurrant anthocyanins seem a possible candidate neuroprotective agent that can be studied for its use in PD patients. The relatively simple composition of blackcurrant anthocyanins makes them convenient to be studied in the CSF of PD patients. Similarly, based on previous lab-, animal- and human- studies, the potent antioxidant effects of anthocyanins should—in theory—help alleviate OS that dopaminergic cells in substantia nigra undergo, as well as enhance endogenous antioxidant defences resulting in less ROS formation (Bub et al., 2003; Cho et al., 2003). Achieving these effects means slowing down the neural degeneration in the substantia nigra and—by extension—neuroprotection.

1.3.5.4 Human metabolism and transport of blackcurrant anthocyanins

Metabolic properties of anthocyanins have been the subject of recent research. This section will concentrate on human metabolism and transport of blackcurrant anthocyanins. However, since some areas in this field have not yet been researched in humans, animal studies will also be occasionally cited.

Gastric and intestinal absorption

Blackcurrant anthocyanins have been shown to be absorbed from the stomach and small intestines in humans (Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999; Nielsen, Dragsted, Ravn-Haren, Freese, & Rasmussen, 2003). Several mechanisms of absorption have been proposed, including permeation through organic anion transporters, such as bilitranslocase—a membrane protein involved in the uptake of bilirubin and other molecules—which is found in the gastric mucosa; however, the exact mechanism is not yet clear (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003). Although blackcurrant anthocyanins are absorbed fairly rapidly (peak plasma concentration reached around 45 minutes post-ingestion), presence of food in the stomach seems to slow this down (plasma concentration peaks around 90 minutes

post-ingestion). Moreover, the absorption rate of blackcurrant anthocyanins has been estimated to be low, varying from 0.007 to approximately 1.4% of ingested dose (Rechner et al., 2002). The authors of the study have attributed this low rate to the inability to estimate or measure the extent of tissue distribution or biliary excretion of these anthocyanins. Blackcurrant anthocyanins appeared in the plasma in their intact forms or as sulfoconjugated forms (Vitaglione et al., 2007). Non-blackcurrant anthocyanins have also been recently detected in human plasma. Healthy participants were fed grape/blueberry juice and smoothies; blood samples were obtained almost every 10 minutes from the time of ingestion. Concentrations peaked at about 60 minutes post-ingestion for C3G and D3G (Kuntz et al., 2015). Of note, participants were only allowed to drink water for three hours after ingestion of the juice or smoothie.

Crossing the BBB

Fewer studies have specifically looked into whether or not anthocyanins cross the BBB. Youdim *et al* have shown that C3R, among other anthocyanins, crossed cell surfaces of ECV304 cells (an *in vitro* model of the BBB). They suggest that some of these anthocyanins diffuse via the paracellular pathway (2003). However, some scientists have criticised the use of ECV304 as a model, since it does not fully mimic the physiological environment of the BBB, particularly in the arena of efflux transporters (Begley, 2004). In animal studies, Talavéra *et al* were able to detect blackberry anthocyanins in the brains of rats (2005). They suggest that anthocyanins could be carried into the CNS by a similar means to that in the gut, namely a bilitranslocase-like transporter.

Urinary and faecal excretion

Absorbed blackcurrant anthocyanins are excreted through the kidneys. Matsumoto *et al* have found that the urine concentrations of the four blackcurrant anthocyanins were 0.11% (\pm 0.05) of the oral dose in the first 8 hours after ingestion (2001). In

contrast to plasma, all forms of blackcurrant anthocyanins (i.e., intact, glucuronidated, methylated and sulfoconjugated) were detected in the urine. This is perhaps because glucuronidated and methylated compounds are formed in the kidney, and hence are directly excreted in the urine (Vitaglione et al., 2007). On the other hand, blackcurrant anthocyanins that are not absorbed from the stomach and small intestines pass into the lower segments of the digestive tract. In the colon, these anthocyanins are broken down, mainly due to the neutral pH in the colon as well as metabolism by colonic microflora (Passamonti et al., 2003). Figure 2-6 shows a scheme of proposed metabolic pathways of cyanidin-glucoside (a family of anthocyanins of which the blackcurrant anthocyanin C3G is a member; Vitaglione et al., 2007).

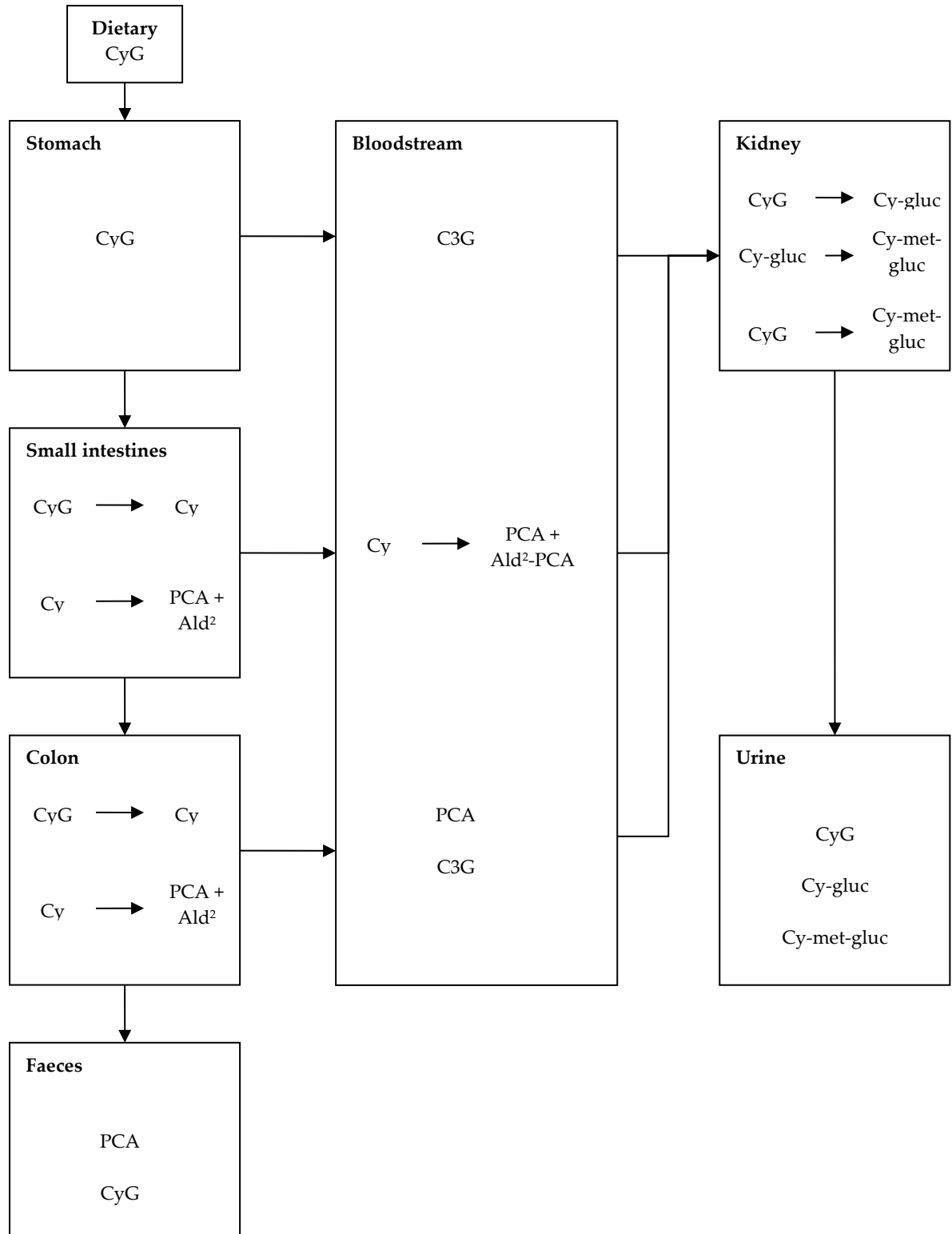


Figure 2-6. Proposed pathways of cyanidin-glucoside metabolism; CyG = cyanidin-glucoside; Cy = cyanidin aglycon; PCA = protocatechuic acid; Ald² = aldehyde; Cy-gluc = cyanidin-glucuronide; Cy-met-gluc = cyanidin-methyl-glucuronide (adapted with permission from Vitaglione et al., 2007).

1.4 Insulin-like growth factor-1 trial

1.4.1 Introduction

1.4.1.1 Background

Insulin-like growth factor-1 (IGF-1), also known as somatomedin C, is a hormone polypeptide produced by the liver (Rinderknecht & Humbel, 1978). Whilst it mainly exerts an endocrine function, it also has autocrine and paracrine targets. As the name suggests, IGF-1 plays pivotal physiological roles in growth and anabolism (see below). A similar hormone, insulin-like growth factor-2 (IGF-2), plays a major role in fetal growth (He et al., 2013); however, further discussion of IGF-2 is beyond the scope of this thesis. The two insulin-like growth factors, their two receptors and seven binding proteins are collectively referred to as the “IGF axis” (Lodhia, Tienchaiananda, & Haluska, 2015).

1.4.1.2 Structure

Structurally homologous to insulin, IGF-1 is made up of 70 amino acids connected by three disulphide bonds (Rinderknecht & Humbel, 1978). The aminoterminal of IGF-1, made up of three amino acids, plays a major role in binding IGF-1 to its binding proteins. If the IGF-1 aminoterminal is treated with an acid protease, it is cleaved to form cyclic glycine-proline (cGP; discussed below; Nilsson-Håkansson et al., 1993; Sara et al., 1993; Yamamoto & Murphy, 1994).

1.4.1.3 Transport around the body

The majority of circulating IGF-1 is bound to one of the insulin-like growth factor-binding proteins (IGFBP). Binding to IFGBPs allows the body to regulate IGF-1, as

only the unbound form of IGF-1 (i.e., “free” IGF-1) is biologically active (reviewed by Binoux, 1995). Therefore, serum concentration of IGF-1 does not necessarily reflect biological activity.

1.4.2 Molecules related to IGF-1

1.4.2.1 IGFBP-3

Upwards of 75% of circulating IGF-1 is bound to IGFBP-3 (Binoux, 1995). Binding to IGFBP-3 prevents it from activating IGF receptors and thus protects IGF-1 from being metabolised (Binoux, 1995). Free IGF-1, on the other hand, is rapidly metabolised or internalised after interacting with IGF receptors. Given the short window of opportunity during which free IGF-1 can be measured, other indices have been suggested (Guan et al., 2015; Sifakis, Akolekar, Kappou, Mantas, & Nicolaides, 2012). The IGF-1/IGFBP-3 ratio is currently being employed as a surrogate for free IGF-1 (i.e., higher IGF-1/IGFBP-3 ratio indicates lower free IGF-1). The reliability of this measure under pathological conditions, however, has been questioned (Sifakis et al., 2012).

1.4.2.2 cGP

Once cleaved, the tri-peptide (i.e., glycine-proline-glutamate) aminoterminal of IGF-1 exists in a *trans:cis* isomeric mixture (80:20); the *cis*-isoform is then further metabolised to form cGP (Nilsson-Håkansson et al., 1993; Sara et al., 1993; Yamamoto & Murphy, 1994). Glycine-proline-glutamate has been demonstrated to elicit the release of acetylcholine and dopamine from murine brain slice cultures, independent of binding to IGF-1-receptors (Nilsson-Håkansson et al., 1993).

The biological activity of cGP itself, however, was not established until later (see review by Samonina, Ashmarin, & Lyapina, 2002). Recently Guan and her colleagues (2014) demonstrated that cGP regulates IGF-1 bioavailability by competitively interfering with IGF-1 binding to IGFBP-3 (see Figure 2-7); they postulate that the cGP/IGF-1 ratio (i.e., higher cGP/IGF-1 indicates higher free IGF-1) may in fact be a superior surrogate for free IGF-1 compared with IGF-1/IGFBP-3 ratio (Guan et al., 2014).

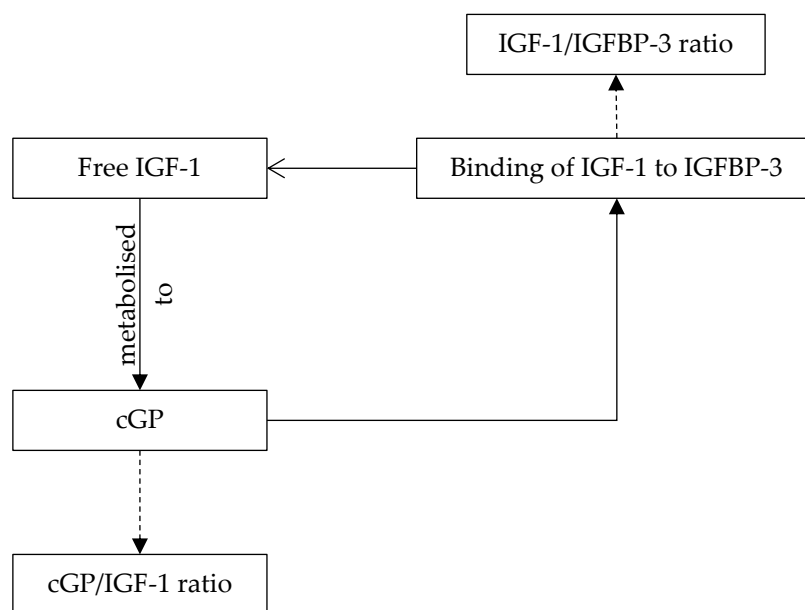


Figure 2-7. The interplay between IGF-1, cGP, IGFBP-3 and the associated ratios; cGP = cyclic guanine-proline; IGF-1 = insulin-like growth factor-1; IGFBP-3 = insulin-like growth factor binding protein-3 (adapted with permission from Guan et al., 2015).

1.4.3 IGF-1 in health and disease

1.4.3.1 General remarks

Whereas IGF-2 is a major growth factor during the fetal period, IGF-1 assumes a bigger growth-promoting role in children and adults. In fact, IGF-1 mediates most of the anabolic effects of growth hormone— these extend to a multitude of cell types including bone, cartilage, haematopoietic and immune, muscle, nerve and splanchnic

visceral cells (Bikle et al., 2015; Christopoulos, Msaouel, & Koutsilieris, 2015; Nieto-Estevez, Defterali, & Vicario-Abejon, 2016).

IGF-1, however, does not act in isolation and is part of an intricate system of several signalling pathways and downstream mediators (Yakar et al., 2002). The cascade of events (with subsequent trophic effects of IGF-1) is mainly triggered by the high-affinity binding of IGF-1 to its receptor, IGF-1-receptor. IGF-1, however, can also bind to other related receptors—including insulin receptors—albeit with lower affinity (Cheng et al., 2000); this may account for the observed hypoglycaemic effect of IGF-1 when given in pharmacological doses (Bang, Polak, Woelfle, & Houchard, 2015).

1.4.3.2 IGF-1 and neuronal health

Perhaps one of IGF-1's best characterised roles is in neuronal growth physiology. It has been demonstrated as a pivotal substance throughout the lifecycle of neurons; this includes trophic effects on developing neurons and inducing differentiation (i.e., neuroproliferation; Russo, Gluckman, Feldman, & Werther, 2005), aiding neuronal repair and survival—even when neurons are damaged—(i.e., neuroprotection; Rabinovsky, 2004) and supporting neuronal development and neuroplasticity (i.e., neuromodulation; Benarroch, 2012; Fernandez & Torres-Aleman, 2012).

1.4.3.3 IGF-1 and neurological dysfunction

The premise of a neuroprotective/neurorestorative effect of IGF-1 holds a certain appeal for its clinical implications. As a result, intense research has been undertaken to explore the underpinnings of IGF-1 and its metabolites for possible bedside applications (Guan & Gluckman, 2009). The two main neurological applications for IGF-1 and its derivatives have been therapeutic uses in acute hypoxic/ischaemic

insults (Guan & Gluckman, 2009) and diagnostic applications (more so in neurodegeneration due to the protracted clinical course; discussed below).

The therapeutic applications of IGF-1 and its active metabolites for neurological disorders remain hampered by pharmacodynamic trepidations and pharmacokinetic impracticalities. For example, not only is central administration of IGF-1 invasive and cumbersome, but it also results in poor neuronal uptake (Pardridge, 1997). Peripheral administration, on the other hand, results in objective neuroprotection in rat brains damaged by hypoxia/ischaemia (Liu, Fawcett, Thorne, & Frey, 2001; Saatman et al., 1997). However, peripheral administration exposes other non-neuronal tissue to IGF-1; given the potent anti-apoptosis effect of IGF-1, there is a theoretical risk of promoting mitogenesis and/or carcinogenesis (especially in pharmacological doses).

1.4.4 IGF-1 in PD

1.4.4.1 Background

Several associations, although preliminary at times, between IGF-1 and PD have been drawn in the literature. Not only is the survival of substantia nigra neurons (including dopaminergic cells) dependent on IGF levels during embryogenesis (Zawada, Kirschman, Cohen, Heidenreich, & Freed, 1996), but they also express a high number of IGF-1-receptors later in life (Mashayekhi, Mirzajani, Naji, & Azari, 2010). Furthermore, metabolites of IGF-1 (including cGP) have been shown to protect dopaminergic cells from 6-hydroxydopamine-induced oxidative injury in murine models of PD (Guan et al., 2000; Krishnamurthi, Mathai, Kim, Zhang, & Guan, 2009; Quesada, Lee, & Micevych, 2008). This effect may be related to IGF-1's known role as a potent inhibitor of programmed cell death (i.e., apoptosis), such that abundance of IGF-1 (and therefore of its metabolites) exerts a neuroprotective effect.

Deficiency of IGF-1 (whether quantitative or qualitative), on the other hand, may accelerate the rate of cellular demise. Whilst a short lifespan for abnormal cells is advantageous (e.g., to curb the promulgation of neoplastic cells; Gallagher & LeRoith, 2011), normal permanent cells (i.e., cells incapable of reproduction such as dopaminergic neurons) may suffer from a short lifespan since they cannot regenerate. This latter observation was the basis of a recent hypothesis linking maternal exposure to bisphenol A (which down-regulates IGF-1 in fetuses) to an increased risk of PD later in life (Huang et al., 2014). Qualitative IGF-1 deficiency or “IGF-1 resistance” (characterised by a blunted end-organ response to IGF-1 and/or impairment of IGF-1 function) has also been implicated in PD progression, namely motor and cognitive decline (Ma et al., 2015; Picillo et al., 2013), although similar results were not reproduced in other studies (see below).

1.4.4.2 IGF-1 as a biomarker for PD

Because of the seemingly intertwined relationship between IGF-1 and PD, the former has been suggested as a biologically plausible biomarker for PD. This has arisen after observing higher plasma IGF-1 concentrations in PD patients than in controls (Chung et al., 2005; Godau et al., 2011; Ma et al., 2015; Numao, Suzuki, Miyamoto, Miyamoto, & Hirata, 2014; Picillo et al., 2013). However, a number of other studies failed to show a significant difference in plasma IGF-1 concentrations in PD patients compared with controls (Numao et al., 2014; Schaefer, Vogt, Nowak, & Kann, 2008; Tuncel, Inanc Tolun, & Toru, 2009).

Added to the inconsistent findings above, several confounders that affect IGF-1 levels preclude its usefulness as a biomarker in PD. These include the known wide inter-individual variability (Verheus et al., 2008), and the effect of age (Godau, Herfurth, Kattner, Gasser, & Berg, 2010; Juul et al., 1994), weight (Faupel-Badger, Berrigan,

Ballard-Barbash, & Potischman, 2009), comorbidities (e.g., diabetes mellitus; Teppala & Shankar, 2010) and medication use (e.g., anti-depressants; Kopczak et al., 2015).

Finally, there is a paucity in the literature on the explicit use of IGF-1 as a biomarker for PD in longitudinal studies (i.e., as a progress and monitoring biomarker; Bernhard et al., 2016; Numao et al., 2014; Picillo et al., 2013). In a recent study by Bernhard and colleagues, patients with moderate PD (disease duration 4-8 years) but not early PD (disease duration ≤ 3.5 years) had significantly higher baseline plasma concentrations of IGF-1 compared with healthy controls (2016). IGF-1 levels were not found to correlate with annual cognitive changes as assessed by the Mini-Mental State Examination (MMSE). The generalisability of these results maybe rather limited, however, due to the arbitrary delineation of disease severity, the small number of participants ($n = 36$) and the use of MMSE rather than other more suitable instruments for cognitive assessment.

1.5 C-type natriuretic peptide trial

1.5.1 Introduction

1.5.1.1 Natriuretic peptides

Natriuretic peptides refer to a family of structurally homologous peptides synthesised by various cells in the body. They act to promote urinary excretion of sodium (i.e., natriuresis) which is physiologically coupled with water excretion (i.e., diuresis) from the ECF compartment. This serves in the body's regulation of its fluid status and mean arterial pressure. Natriuretic peptides exert their effects mainly by binding to one of several natriuretic peptide receptors (NPR). Members of this natriuretic peptide family include atrial natriuretic peptide (ANP), B-type natriuretic

peptide (BNP), C-type natriuretic peptide (CNP, discussed in detail below), and more recently, dendroaspis natriuretic peptide ('DNP', Schirger et al., 1999) and urodilatin (Schulz-Knappe et al., 1988).

To understand the function of these natriuretic peptides, it is useful to consider the nomenclature used in the endocrinology literature. The suffix –“crine” (εκκρίνει Gr.) denotes the process of secretion. Secreted substances (e.g., peptides) function to alter the recipient cell's behaviour and physiology. Depending upon the location of recipient cells, different prefixes are used. From proximal to distal, these include: intracrine signals (i.e., substances that act inside the cell in which they were produced without physically leaving the cell), autocrine signals (i.e., substances that act on the cell in which they were synthesised by binding to receptors on its outer membrane), juxtacrine signals (i.e., these affect cells/structures physically contacting the cell which produced the juxtacrine molecules), paracrine signals (i.e., substances that affect nearby local cells) and finally endocrine signals (i.e., molecules that travel in the circulation to a distant target).

1.5.1.2 ANP and BNP

ANP is perhaps the most extensively studied natriuretic peptide. It is mainly produced by and stored in cardiac myocytes in the atria (Nemeh & Gilmore, 1983; Sonnenberg, Chong, & Veress, 1981). Atrial myocytes release ANP in response to excessive atrial stretch (e.g., by hypervolaemia) in order to lower the circulatory volume and restore fluid homeostasis (Lopez et al., 1995; Nemeh & Gilmore, 1983). ANP achieves this by binding to type-A NPR (NPR_A) found in the vascular smooth muscle and kidney tubules (i.e., endocrine action), which leads to vasodilation and promotes urinary sodium and water excretion (Lopez et al., 1995).

BNP was previously named the brain natriuretic peptide as it was first identified in porcine brain extracts (Sudoh, Kangawa, Minamino, & Matsuo, 1988). In humans, however, BNP is largely synthesised by ventricular cardiac myocytes (Takahashi, Allen, & Izumo, 1992). Much like ANP, BNP is also released in response to cardiac stretch in which case it promotes natriuresis by binding to NPR_A (Kambayashi et al., 1990).

1.5.2 CNP structure and synthesis

CNP is synthesised as a large prohormone form (i.e., proCNP) by a variety of tissue cells around the body (Tawaragi et al., 1991). It then undergoes modification by the endoprotease enzyme, furin, the process of which yields two products: CNP-53 (also referred to as the aminoterminally extended form) and an aminoterminal fragment, NTproCNP (Wu, Wu, Pan, Morser, & Wu, 2003). NTproCNP was first isolated and studied in Christchurch, New Zealand by Prickett and his colleagues (2001). CNP-53 is further processed by an unknown mechanism, perhaps an exopeptidase, to produce CNP-22 and another aminoterminal fragment, which is yet to be identified. Although limited literature pertaining to the identity of this aminoterminal fragment being NT-CNP-53 exists (Komatsu et al., 1991; Sudoh, Minamino, Kangawa, & Matsuo, 1990), the radioimmunoassay method utilised has not been validated and is deemed too insensitive, both reasons of which abrogate confidence in the generated data (Prickett et al., 2001).

CNP -53 and -22 are the biologically active forms, whilst the functions of NTproCNP (and NT-CNP-53) are yet to be elucidated (Minamino, Kangawa, & Matsuo, 1990; Pemberton, Siriwardena, Kleffmann, & Richards, 2014). Vlachopoulos *et al* have recently demonstrated a relationship between NTproCNP and indices of arterial function (2010); however, NTproCNP was used as a proxy for CNP, rather than being

the regulator of arterial function *per se*. See Figure 2-8 for a visual representation of the CNP molecule and its synthesis pathway.

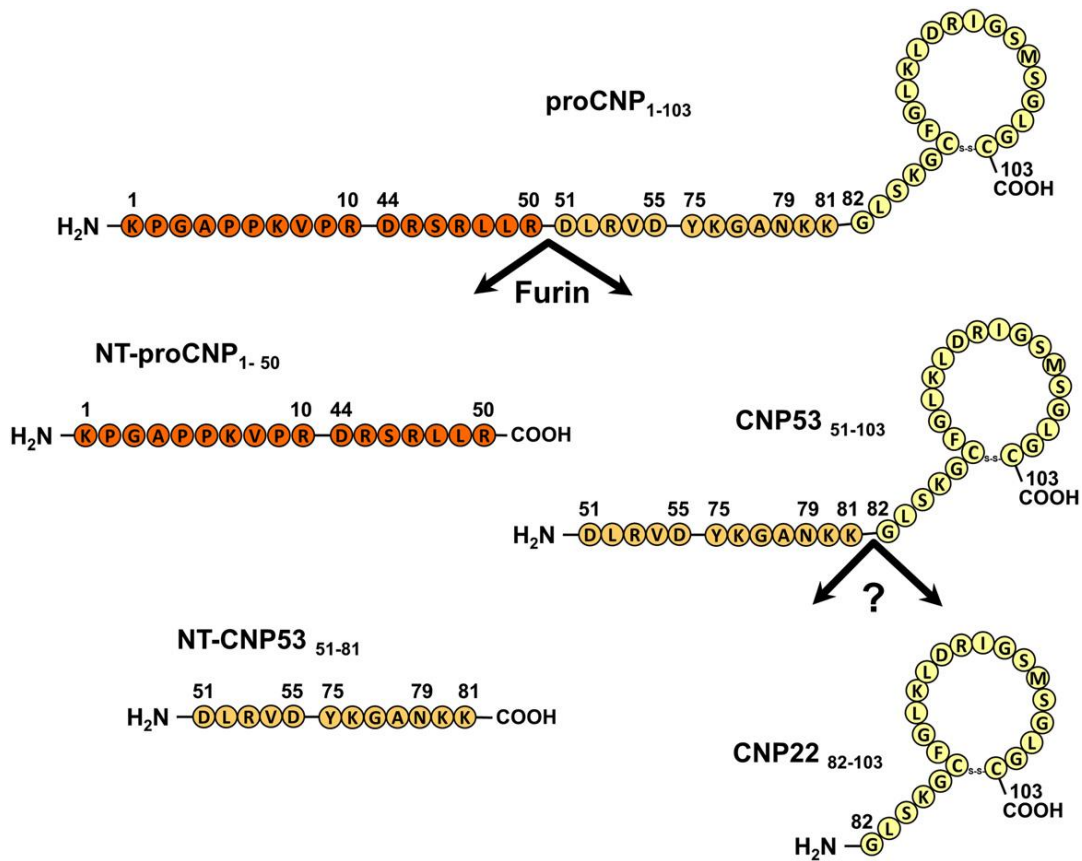


Figure 2-8. The pathway of proCNP processing—initially by the intracellular enzyme, furin, to produce NTproCNP and CNP. Downstream processing, by an unknown mechanism, results in the bioactive CNP-22 (reproduced with permission from Zakeri et al., 2013).

1.5.3 CNP metabolism and degradation

Part of the reason why CNP only exerts its effects locally is the rapidity with which it is cleared from the circulation after secretion. Once intracellular proCNP is processed by furin, the two products, CNP-53 and NTproCNP are secreted across the cell membrane in equimolar amounts (Schouten et al., 2011). Whereas the catabolic fate of NTproCNP is largely still unknown (Schouten et al., 2011), the biologically active forms of CNP (i.e., CNP-53 and its derivative, CNP-22) are swiftly cleared by three substances; neprilysin-1, type-C NPR (NPR_C) and insulin-degrading enzyme.

Neprilysin-1 is a membrane-bound zinc-binding metalloprotease (i.e., an enzyme whose catalytic activity involves a metal; Kenny, Bourne, & Ingram, 1993). Neprilysin-1 is known by many other names including neutral endopeptidase-1 (NEP-1), cluster of differentiation 10 and enkephalinase (Thong et al., 2014). NEP-1 appears to not only hydrolyse CNP, but also ANP, BNP, urodilatin and other peptides and receptors (Kenny et al., 1993). NEP-1 is highly expressed in both the CNS and elsewhere. In rats, NEP-1 has been found in the basal ganglia, choroid plexus, dentate gyrus, ependyma, hippocampus, hypothalamus, olfactory bulb and tubercle and the spinal cord's superficial laminae of the dorsal horn (Facchinetti, Rose, Schwartz, & Ouimet, 2003). With ageing, murine brains expressed less NEP-1 production and activity (Higuchi, Iwata, & Saido, 2005). In humans, NEP-1 has been found to be expressed in many tissues, including adipose (Katsuda et al., 2013), brain (Mazur-Kolecka & Frackowiak, 2006), liver (Dragovic, Deddish, Tan, Weber, & Erdos, 1994) and testicular tissues (Thong et al., 2014). Similar to aged murine brains, CSF of patients with early AD has been reported to contain reduced levels of NEP-1 (Maruyama et al., 2005).

NPRs are a family of receptors that bind the various natriuretic peptides. One classification system of NPRs is whether the receptor activates guanylyl cyclase (GC) or not (Anand-Srivastava, 2005). GC-activating receptors include NPR_A and B-type NPR (NPR_B); NPR_A primarily binds to ANP and, with about 10 times less affinity, to BNP (Moya et al., 1998)—meanwhile NPR_B is the primary receptor for CNP (discussed in the following section). Activation of the GC pathway leads to a cascade of events the end-result of which is often a stimulatory response. NPR_C, on the other hand, is considered a non-GC activating receptor (Matsukawa et al., 1999). Its affinity to the three most studied natriuretic peptides, in descending order, is: ANP, CNP and then BNP (Suga et al., 1992). NPR_C is the most widely expressed in the body (including neural tissue) of all NPRs (Matsukawa et al., 1999). Its principal role is the uptake and internalisation of natriuretic peptides (Nussenzveig, Lewicki, & Maack,

1990). In fact, NPR_C was thought of being a “clearance receptor” for a period of time (Anand-Srivastava, 2005). However, it is now known NPR_C is also implicated in several other physiological functions, including the generation of inhibitory signals through second messengers (Anand-Srivastava, Sairam, & Cantin, 1990; and see review by E. R. Levin, 1993).

Insulin-degrading enzyme is also known as insulysin, and akin to NEP-1, is a large zinc-dependent metalloprotease (Shen, Joachimiak, Rosner, & Tang, 2006). Whilst the function of insulin-degrading enzyme has most extensively been described in ANP metabolism (Müller, Schulze, Baumeister, Buck, & Richter, 1992), a role in CNP degradation has also been described. *In vitro* studies have demonstrated reduced expression of insulin-degrading enzyme led to increased CNP-induced activation of NPR_B (i.e., consistent with CNP inactivation) and vice versa (Müller et al., 1992; Ralat et al., 2011). The presence of insulin-degrading enzyme within the natriuretic peptide milieu also resulted in paradoxical effects on receptor activation, especially by BNP (Potter, 2011). It appears, therefore, that insulin-degrading enzyme modulates potency and receptor preference, in descending order, of: ANP, CNP and BNP (Potter, 2011).

Finally, a few other mechanisms of clearing CNP have been recently proposed. For example, Whyteside and colleagues recently identified neprilysin-2 in human brain tissue (2008). Whether neprilysin-2, or other suggested mechanisms, preferentially catabolise CNP as a substrate remains to be elucidated.

1.5.4 CNP function

1.5.4.1 Background

While ANP and BNP seem to affect the body in similar ways, evidence shows that CNP is different. Unlike ANP and BNP's endocrine functions, CNP appears to act locally (i.e., autocrine and paracrine functions). Furthermore, CNP binds to NPR_B (Koller et al., 1991) as opposed to ANP and BNP binding to NPR_A (see Figure 2-9). This, in part, accounts for the weak natriuretic effect of CNP compared with ANP or BNP (Canaan-Kuhl, Jamison, Myers, & Pratt, 1992).

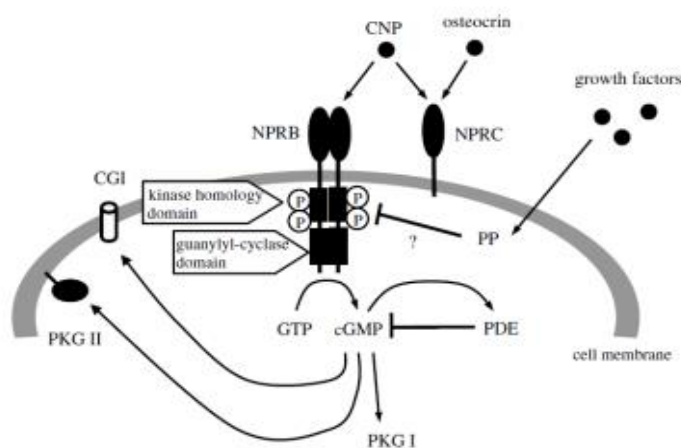


Figure 2-9. Anatomy of NPR_B; the function of the receptor is discussed below (reproduced with permission from Pejchalova, Krejci, & Wilcox, 2007).

The full range of functional roles of CNP in healthy individuals is yet to be determined. However, CNP has been implicated in several developmental and physiological pathways. Below is a discussion on CNP's functions according to site (the nervous system is considered separately and in more detail in the following sections.)

1.5.4.2 Cardiovascular system

As mentioned earlier, contrary to the endocrine functions of ANP and BNP, CNP operates on autocrine and paracrine levels. Therefore, it is logical to assume that CNP does not play a significant role in controlling the body's systemic blood pressure (i.e., mean arterial pressure); this deduction has been corroborated by findings by Lopez *et al* (1997). Rather, CNP appears to act upon microcirculations (e.g., coronary blood vessels, Wiley & Davenport, 2001), causing local vasodilation. CNP achieves this by hyperpolarising (and therefore relaxing) vascular smooth muscle cells—hence, referring to CNP as an endothelial-derived hyperpolarising factor (Chauhan *et al.*, 2003). Perhaps more clinically-promising is CNP's ability to inhibit cardiac and vascular remodelling post-injury (e.g., post-myocardial infarction, Soeki *et al.*, 2005). CNP is believed to curtail cardiac myocyte hypertrophy, smooth muscle proliferation and tissue fibrosis (Bouchie, Hansen, & Feener, 1998). Such function has also been implicated in CNP's role as an anti-atherogenic agent (Casco *et al.*, 2002).

1.5.4.3 Endochondral bone

Perhaps one of the most studied arenas of CNP physiology is its involvement in long bone growth (Peake *et al.*, 2014). CNP regulates cell growth and differentiation, ultimately leading to endochondral ossification (i.e., the essential process underlying long bones formation and growth; Olney, 2006). In mice, mutations knocking out genes encoding CNP (Chusho *et al.*, 2001; Fujii *et al.*, 2010) or NPR_B (Tsuji & Kunieda, 2005) result in a dwarf phenotype and skeletal dysplasia. In humans, CNP has been suggested to play an integral role in contributing to a person's height. To that effect, a genetic mutation of NPR_B results in extreme short stature (a condition called acromesomelic dysplasia, type Maroteaux; Bartels *et al.*, 2004), while high CNP production/signalling is believed to contribute to the tall phenotype of some Northern European populations (Estrada *et al.*, 2009).

1.5.4.4 Reproductive organs

CNP also appears to be critical for normal reproductive organ function. What is interesting is that CNP is pivotal for both reproductive organogenesis (i.e., organ development) in an embryo as well as maintaining normal sexual function in an adult in both sexes.

In female mice, the non-pregnant uterus and ovaries contain more CNP messenger RNA than any other organ (Stepan, Leitner, Bader, & Walther, 2000). In fact, CNP was found to be essential for ovarian development and function (including the maintenance of oocyte meiotic arrest; Yang, Wei, Ge, Zhao, & Ma, 2016), and NPR_B-deficient mice were found to develop ovarian hypoplasia (Tamura et al., 2004). Moreover, uteri of non-pregnant rats contain a hundred times more NPR_B (i.e., CNP receptor) than NPR_A (Dos Reis et al., 1995). During pregnancy, CNP messenger RNA expression increases several-fold in the murine uterus, but decreases significantly in the ovaries (Stepan et al., 2001). It is hypothesised that the increase in CNP has a muscle-relaxing effect which protects the gestation by sidestepping premature uterine contractions (Drewett, Fendly, Garbers, & Lowe, 1995). The dramatic decrease in ovarian CNP, on the other hand, is thought to reflect the gestational decline in luteinising hormone levels (which controls ovarian CNP production), (Jankowski et al., 1997). In healthy pregnant female humans, CNP and NRP_B were detected in reproductive organs, more in the placenta than in the uterus (Stepan, Faber, Stegemann, Schultheiss, & Walther, 2002). Intriguingly however, the pattern was reversed (i.e., more CNP in the uterus and less in the placenta) in gestations complicated by pre-eclampsia and intra-uterine growth restriction (Stepan, Faber, Stegemann, et al., 2002; Stepan, Faber, & Walther, 2002).

In males, CNP has been implicated in normal testicular function; it appears to modulate murine blood-testis barrier function (Xia, Mruk, & Cheng, 2007) as well as

contribute to molecular signalling in porcine seminal plasma (Chrisman, Schulz, Potter, & Garbers, 1993). In humans, Middendorff *et al* have shown that CNP and NPR_B are present in the human testis (1997). In another study, the same group reviewed the several functional roles of testicular CNP, including regulating germ cell development, spermatozoon motility and testosterone synthesis (see Middendorff *et al.*, 2000). In the penis, CNP is thought to contribute to erectile function, after NPR_B was found to be expressed in human corpus cavernosal tissues (Kütke *et al.*, 2003). To that effect, patients with vascular erectile dysfunction were found to have lower NTproCNP levels compared with controls (Vlachopoulos *et al.*, 2009).

1.5.5 CNP and the nervous system

1.5.5.1 Presence of CNP in the CNS

CNP is the most abundant of all natriuretic peptides in the CNS, at least in the case of rats and humans (Komatsu *et al.*, 1991). Plasma levels of CNP-22 and NTproCNP increased when arterial versus venous samples across the head and neck (i.e., an arteriovenous gradient) were examined in sheep (Charles *et al.*, 2006) and humans (Palmer, Prickett, Espiner, Yandle, & Richards, 2009). Whether this represents CNS contribution to the systemic pool of CNP is controversial, however. This is because the same research group has found an inverse relationship between plasma and CSF levels of CNP-22 in human subjects before undergoing non-acute orthopaedic surgery (Schouten *et al.*, 2011). These apparently contradicting results may be explained using the following argument—the physiological condition of the subjects in the two human studies is likely to have been different. Patients in the study by Palmer *et al.* would have likely been sedated, but perhaps not anaesthetised, for the procedure before samples were obtained (2009); CSF turnover is known to increase during sleep (see review by Johanson *et al.*, 2008), which could theoretically account for a ‘cerebral’ source explaining the step-up in the arteriovenous gradient. Samples from patients in

the study by Schouten *et al.*, on the other hand, were obtained from awake patients before they were anaesthetised (2011). The other possibility is that 'non-cerebral' sources contributed to the CNP pool in the sampled returning venous plasma; these could include the eyes (Fernandez-Durango, Nunez, & Brown, 1995) or the cervical dorsal root ganglia (Kishimoto et al., 2008).

1.5.5.2 CNP in health

The exact role of CNP within the CNS is not completely understood. To date, however, CNP appears to be implicated in different physiological pathways rather than exerting a single overarching effect.

In mice, natriuretic peptides, including CNP, have been shown to modulate axonal branching and growth in several areas of the embryonic brain and spinal cord (Zhao & Ma, 2009). In rats, CNP has been shown to be critical in the development of neural progenitor cells, which ultimately lead to perinatal neurogenesis (Müller et al., 2009). Moreover, Decker *et al* have demonstrated a role for CNP in regulating hippocampal synaptic plasticity and influencing hippocampal interneurons in such a manner that it may potentially aid in memory formation (2010). Finally, in a model of bovine BBB cells, CNP has recently been found to significantly influence the expression of zonula occludens-1 (i.e., a type of tight junctions expressed in the BBB), which thence led to increased BBB permeability (Bohara et al., 2014). It is still too early to extrapolate such putative effects of CNP on human BBB, however (see review by Guo, Barringer, Zois, Goetze, & Ashina, 2014).

Studies on CNP and the human CNS have been less forthcoming. Togashi and colleagues confirmed the presence of CNP in human brain and CSF (1992). Furthermore, they found CNP-53 to be the predominant form in the brain, while CNP-22 was the predominant form in CSF (Togashi et al., 1992). Whether this

indicates differential processing of CNP in the human CNS remains a conjecture. Moreover, Kaneko *et al.* measured CNP levels in the CSF of 15 neurologically intact subjects. They found a mean CNP concentration of 2.13 (\pm 0.27) pmol/l (1993). Modestly higher concentrations were found in another cohort of 51 healthy orthopaedic patients 7.9 (\pm 3.2) pmol/l, although one subject had PD and another had diabetic neuropathy (Schouten *et al.*, 2011).

1.5.5.3 CNP in patients with non-PD neurological disorders

A few studies have examined the role of CNP in the pathogenesis of, as well as being a biomarker for, AD. The biologically active forms of CNP (i.e., CNP-53 and CNP-22) have exposed hydrophobic regions (Kourie & Henry, 2002). This structural configuration makes these peptides particularly “sticky” and facilitates their binding to amyloid (Kourie & Henry, 2002). Moreover, NEP-1—shown to decrease with age (Higuchi *et al.*, 2005)—is one of the best characterised enzymes that degrade amyloid- β ('A β ', Hersh & Rodgers, 2008). In fact, NEP-1-overexpressing murine brains exhibited less A β and the mice performed better on behavioural tasks (Marr *et al.*, 2003). Taken together, these findings suggest a possible clinical utility for CNP and/or NEP-1 in the diagnosis (i.e., distinction from other causes of dementia) and prognostication of AD. In fact, Maruyama and colleagues have demonstrated reduced NEP-1 activity in CSF of patients with early AD (2005). Further studies are required to delineate the potential of CNP or NEP-1 as biomarkers for AD.

Several other human studies have tried to ascertain a role of CNP in various neurological disorders. In the sample studied by Kaneko *et al.*, 15 patients had a variety of neurological disorders (e.g., subarachnoid haemorrhage and traumatic brain injury 'TBI'); CSF levels of CNP did not differ in this group compared with normal controls (1993). On the other hand, another Japanese group has shown that CNP levels in the CSF have indeed risen in the acute setting (i.e., within 24 hours) of

subarachnoid haemorrhage; plasma CNP levels remained steady, however (Ikeda, Ikeda, Onizuka, Terashi, & Fukuda, 2001). In patients with TBI, lower plasma CNP levels were predictive of ensuing sepsis (Bahrami et al., 2010).

Finally, there has also been an interest in CSF levels of cyclic guanosine monophosphate (cGMP, a downstream secondary messenger of CNP-induced activation of NPR_A, NPR_B and nitric oxide pathways). The value of CSF cGMP in neurodegenerative disorders remains controversial, however. For instance, whereas one study found lower CSF concentrations of cGMP in amyotrophic lateral sclerosis (ALS) patients (Ilecka, 2004), another study failed to replicate this finding (Oeckl et al., 2012).

1.5.5.4 CNP and PD

In the murine brain, CNP (Langub, Watson, & Herman, 1995) and its receptor, NPR_B (Herman, Dolgas, Rucker, & Langub, 1996), are highly expressed in the mesencephalon where dopaminergic neurons originate. Moreover, CNP has been shown to blunt dopamine release in response to cocaine given to rats (Thiriet et al., 2001). To date, however, hardly any human studies have investigated the role of CNP in PD.

1.6 Exosome trial

1.6.1 Introduction

Extracellular vesicles have garnered recent interest as promising biomarkers for a plethora of disease processes. Excreted by normal and pathological cells, these 20-

1 000 nm vesicles serve, among other functions, as a way of cell-to-cell communication (van der Pol et al., 2014). This communication may be external (i.e., interaction between vesicular membrane protein and recipient cell membrane protein) or internal (i.e., internalisation of the vesicular content by the recipient cell); the content of these vesicles reflect specific traits of the cell of origin.

Based upon size and function, these extracellular vesicles have been classified in the literature into two to six groups – depending on the reporting study. As will become apparent below, a consensus on naming or classifying these vesicles remains lacking to date. There is, however, reasonably sufficient evidence to support classifying extracellular vesicles into four categories: apoptotic vesicles, exosomes, membrane particles and microvesicles. Table 2-2 summarises key details of each class of vesicles.

Table 2-2. The four major classes of extracellular vesicles.

	Apoptotic vesicles	Exosomes	Membrane particles	Microvesicles
Size (nm)	1 000-5 000 (Hristov, Erl, Linder, & Weber, 2004)	30-100 (Heijnen, Schiel, Fijnheer, Geuze, & Sixma, 1999)	50-600 (Marzesco et al., 2005)	20-1 000 (Heijnen et al., 1999)
Shape	Multiple (Hristov et al., 2004)	Cup-shaped (Heijnen et al., 1999)	Cup-shaped (Marzesco et al., 2005)	Cup-shaped
Cell(s) of origin	Most cell types	Most cell types	Epithelial cells only (Marzesco et al., 2005)	Most cell types
Content	DNA and histones (Hristov et al., 2004)	Multiple-see below	CD133 (Marzesco et al., 2005)	?RNA (Ben-Dov, Whalen, Goilav, Max, & Tuschl, 2016)
CD133 = cluster of differentiation 133; DNA = deoxyribonucleic acid; RNA = ribonucleic acid.				

Of relevance to the biological markers trial are the exosomes, small membrane-enclosed vesicles with a size distribution of 30-100 nm (Heijnen et al., 1999). Exosomes were first discovered in the 1980s by Dr Rose Johnstone, of McGill

University, and colleagues during their work on erythrocyte maturation (Johnstone, Adam, Hammond, Orr, & Turbide, 1987).

1.6.2 Nomenclature

The suffix -some (from *sôma* or body, $\sigma\tilde{\omega}\mu\alpha$ Gr.) is often attached to various terms referring to cellular organelles. *Endosomes*, for example, are membrane-bound vesicles located *intracellularly* and that substantially vary in size. The term “exosome” itself reflects the similarity between the vesicular extrusion of exosomes and reverse endocytosis (see Biogenesis below)—this was coined by Johnstone. Descriptive terminology attached to exosomes, however, has been confusing. One system names the vesicles based upon the source from which they are obtained. For example, epididimosomes are obtained from semen, whereas oncosomes are derived from cancerous cells. The other system employs methods of isolation and mechanisms of biogenesis to make the distinction between the different extracellular vesicles.

1.6.3 Biogenesis

The synthesis pathway of exosomes is intricate and complicated—the details of which are beyond the scope of the biological markers study. In brief, contents of the intracellular endosomes destined to be exported out of the cell are packaged into 30-100 nm vesicles. During this multi-step process, the membrane of these packaging vesicles acquires several proteins, including cluster of differentiation antigen 63 (CD63)—which has been utilised as a marker for exosomes.

The formation of the package bodies results in the presence of multiple vesicles within the endosome itself, which is why it is also referred to as a multi-vesicular body (see Figure 2-10). If the membrane of the endosome fuses with the plasma membrane of the cell, the vesicular contents are excreted out of the cell, and are called

exosomes. Once released, such vesicles may be taken up by the same secreting cell, a neighbouring cell or a distant cell; akin to previously described autocrine, paracrine and endocrine signals, respectively.

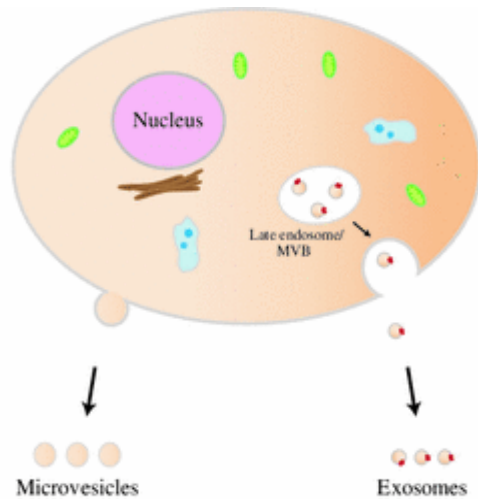


Figure 2-10. Pathway of exosome biogenesis according to Akers *et al* (reproduced with permission 2013); MVB = multi-vesicular body.

1.6.4 Isolation of exosomes

The classic method of isolating extracellular vesicles has been differential centrifugation and ultracentrifugation. They are then classified into different categories according to size, density and expression of protein markers. This approach, however, has recently proven problematic for several reasons (Colombo, Raposo, & Thery, 2014).

First, the utility of traditional centrifugation and ultracentrifugation methods is limited since neither technique lends itself as amenable for high-throughput screening, often required in clinical applications. Furthermore, it is now known that a single cell may produce different extracellular vesicles, some of which share characteristics and functions. Finally, using protein markers to classify exosomes may

be challenging as such proteins are often found in cells of multiple lineages. In the case of CD63, for example, its presence has been documented in vascular endothelium, leucocytes and platelets. Whether CD63 can, thus, serve as a reliable marker for exosomes remains debatable, and further studies are essential to determine the specificity and sensitivity of CD63 for exosomes.

The International Society for Extracellular Vesicles, as a result, has expressed concern over the quality of published literature utilising these methods and has, therefore, published guidelines to standardise extracellular vesicle isolation methods. Colombo and colleagues have recently reviewed the various methods employed and the evidence behind each (2014).

1.6.5 Exosomes in the CNS

1.6.5.1 Physiological processes

Exosomes have been described in a number of processes—both physiological and pathological—within the CNS. Exosomes have been implicated in normal neuronal development, as demonstrated by *in vitro* secretion of exosomes by cortical and hippocampal neurons (Akers et al., 2013). In response to nerve injury, Schwann cell-derived exosomes have been shown to stimulate axonal healing and neuronal regeneration (Lopez-Leal & Court, 2016). Moreover, exosomes serve as a communication vehicle between different neurons (Schiera, Di Liegro, & Di Liegro, 2015). For instance, Frühbeis *et al* have documented exosome-mediated oligodendrocyte-to-neuron, and vice versa, communication (2013). Not only that, but exosomes released from oligodendrocytes were also found to contain neuroprotective substances (glycolytic enzymes, proteins and RNA) when signals were generated from distressed neurons (Schiera et al., 2015).

Even beyond the confines of the CNS, exosomes were found to have transferred genetic material from haematopoietic cells and Purkinje neurons, implying a neuro-immune signalling response in inflammatory states (Ridder et al., 2014), and providing indirect evidence that peripheral exosomes are able to cross the BBB. More direct evidence comes from peripherally injected exosomes which were found to cross murine BBB and to be present in neurons (Alvarez-Erviti et al., 2011).

1.6.5.2 Pathological processes

In contrast to the described neuroprotective function of exosomes, they have also been suggested to perpetuate neurodegeneration in certain pathological processes. Exosome-mediated transfer of misfolded proteins (e.g., mutated α -synuclein protein in PD) has been shown to occur from diseased cells to the extracellular milieu and to normal cells (Stuendl et al., 2016). Indeed this propagation pattern is an aetiological characteristic of several of the neurodegenerative disorders which progress along distinct neuroanatomical pathways.

1.6.5.3 Exosomes in PD

In serum samples obtained from PD patients (idiopathic and genetic), the most common type of microsomes were found to be exosomes; these exosomes did not differ in their size or concentration compared with samples obtained from healthy subjects (Tomlinson et al., 2015). However, analysis of the content of the exosomes from PD sera revealed characteristic protein changes. Twenty-three “exosomal” proteins were found much more abundantly in serum samples from PD patients compared with normal subjects and ALS patients (see Tomlinson et al., 2015 for detailed protein analysis). Furthermore, some of these proteins are known to possess antioxidant and/or chaperone properties (Tomlinson et al., 2015) which implies function-specific adaptation of exosome packaging in response to the disease state

(i.e., PD). Such peripheral (i.e., non-CNS) changes to exosomes have been described in PD (Besong-Agbo et al., 2013; Scherzer et al., 2007), although their significance is still debatable.

Alpha-synuclein-containing exosomes were found to be in significantly higher concentrations in plasma of PD patients compared with controls (Shi et al., 2014); the investigators also found a significant correlation between exosomal α -synuclein concentration in plasma and PD severity (Shi et al., 2014). The same study by Shi and colleagues (2014), as well as other recent *in vivo* studies (Kunadt et al., 2015), have demonstrated the presence of α -synuclein in CSF exosomes in PD patients. However, unlike plasma, measurement of exosomal α -synuclein in CSF did not reveal any clinically meaningful correlations with PD status (Kunadt et al., 2015; Shi et al., 2014).

1.7 Human eye movements

1.7.1 The purpose of human eye movements

Human saccadic eye movements serve a basic function: to rotate the eyes towards an object of interest so as to allow the brain to perceive it and its surrounding environment. The goal is to ensure that the image of the object of interest falls on the fovea centralis, where visual acuity is at its sharpest and colour sensitivity is maximal. Because the body and the object can be stationary or in motion, an intricate system of other eye movement types exists also (Swenson, 2006).

1.7.2 Types of human eye movements

Five types of eye movements fall within one of two functional classes (Anderson & MacAskill, 2013). The first class of eye movements serves to swiftly bring the *fovea*

centralis to bear on the object of interest. This primarily requires a fast 'jump' of the eyes from a previous target to the newly acquired target. Such eye movements are referred to as saccades (Vilis, Hepp, Schwarz, & Henn, 1989; discussed in more detail in the following section). Once the object of interest is fixated, the second class of eye movements tries to stabilise the fovea on the object of interest regardless of body or object motion. To achieve this, four other types of eye movements are utilised, which are: optokinetic nystagmus (OKN), smooth pursuit, vergence (convergence and divergence) and vestibulo-ocular reflexes (VOR; rotational and translational).

To illustrate the different types of eye movements, consider the following example: the buzzing sound of a nearby bee reflexively catches the attention of a man reading a book and he rapidly shifts his sight towards it (i.e., saccades). As the bee flies from side to side, he follows its movements (i.e., smooth pursuit). If the bee is joined by a swarm of bees flying from side to side, OKN allows his eyes to follow the swarm (via a combination of saccadic and pursuit eye movements). Alternatively, if the bee flies away from him into the distance, his eyes will diverge as it moves further and further. If the bee flies towards him, however, his eyes will converge as it moves closer and closer. If the bee flies very close to him, he may try to avoid it by moving his head to one side while still looking at it. The VOR compensates for head movements by generating eye movements of equal magnitude but in the opposite direction (Swenson, 2006).

1.7.3 Saccadic eye movements

1.7.3.1 Introduction

As briefly noted in the previous section, saccades are rapid eye movements that are often described as jump- or jerk- like movements of the eyeball (Yarbus, 1967; pp. 129-

147). Saccades are so rapid that, combined with other central processes, we cannot see during the movement (Swenson, 2006).

1.7.3.2 Types

Types of saccadic eye movements can be grouped into one of two categories, based upon the setting in which each type is more likely to occur. The first category – which encompasses eye movements that are more likely to occur naturally (i.e., in real life)—includes memory-guided saccades, microsaccades and reflexive saccades (Anderson & MacAskill, 2013). Memory-guided saccades refer to saccadic eye movements made towards a location where, from memory, the required information is most likely to be. For example, a Windows® PC user automatically looks at the top right corner of the screen to locate the X button when trying to close an active Word document. Microsaccades are smaller jumps of the eye that occur spontaneously upon ocular fixation (Swenson, 2006). They are thought to be essential to save visual perception from fading (Martinez-Conde, Macknik, & Hubel, 2004), to correct fixations (Otero-Millan et al., 2011), as well as being involved in other attention and perception functions (see review by Martinez-Conde, Macknik, Troncoso, & Hubel, 2009). Lastly, reflexive saccades, perhaps the most intuitive to understand, refer to eye movements made when a new target appears at a random location eccentric to a previous point of fixation (Anderson & MacAskill, 2013). The man in our previous example made a reflexive saccade shifting his eyes from the book to the bee. A fourth type of saccadic eye movements, spontaneous saccades, is sometimes grouped with this category. It refers to ‘aimless’ saccades made during another activity (e.g., speech) or while resting in the dark before falling asleep (Pierrot-Deseilligny, Rivaud, Gaymard, Muri, & Vermersch, 1995).

The other category involves saccadic eye movements that are much less likely to be experienced outside laboratory settings. This category includes antisaccades and

delayed and predictive saccades. Antisaccades denote the action of suppressing looking at a target and instead making a saccade in the opposite direction. Predictive saccades, on the other hand, refer to saccades between two targets alternating between fixed positions at a fixed rate (Anderson & MacAskill, 2013). Such eye movements, albeit artificial, can be particularly helpful in behavioural psychology research (Bridgeman, 1992).

1.7.3.3 Definitions

In the following sections and chapters, a few saccade-related parameters will be mentioned repeatedly. It is helpful, therefore, to establish a definition of each of these parameters (Wilson, Glue, Ball, & Nutt, 1993). Saccade amplitude refers to the size of the saccade, which in turn determines the accuracy of the saccade. Amplitude is frequently measured in degrees or, less frequently, in arcminutes (i.e., $1^\circ = 60$ arcminutes). Non-angular measures of amplitude may also be used (e.g., eye movements during reading), but these are often used in specific fields of study unrelated to work presented here. A related parameter to amplitude is gain. The gain is the ratio of the participant's saccade amplitude divided by the desired saccade amplitude (i.e., target eccentricity). Under ideal conditions, this ratio is approximately one or slightly less; deviation from this norm is referred to as saccade dysmetria. Thus, gains greater than one denote a hypermetric saccade (i.e., one that is too large), whereas gains less than one signify a hypometric saccade (i.e., saccade that is too small).

Saccade duration is the total time it takes to complete the saccade. Most saccades are completed within tens of milliseconds (ms). Saccade latency refers to the time it takes from the appearance of a target to the onset of a saccade in response to that target. Unlike other saccade parameters, latency is tremendously variable (typically 70-350 ms). Finally, saccade peak velocity is the highest speed reached during a saccade.

Slowed saccades have reduced velocities, but can still be fairly accurate (Beydagi, Yilmaz, & Suer, 1999).

1.7.3.4 Neural pathway

The neural network underlying human saccadic eye movements is complex and involves multiple cortical and subcortical structures. To complicate matters further, each specific saccade type has a partially differing neural circuit within the network. To help understand the overall organisation of such a complex system, it is useful to start with 'the big picture'; that is, an area triggers a signal which may be modulated by various other areas within the brain before an oculomotor response (i.e., a saccade) is generated.

There are three major cortical areas that directly trigger saccades when stimulated, and at least two other major areas involved in the preparation of saccades. Each area appears to specialise, albeit partially, in triggering a specific type of saccade or a specific amplitude of saccadic eye movement (Pierrot-Deseilligny et al., 1995). Areas that directly trigger saccades are the frontal eye field ('FEF'; Petit et al., 1993), the parietal eye field (Muri, Iba-Zizen, Derosier, Cabanis, & Pierrot-Deseilligny, 1996) and the supplementary eye field ('SEF'; Paus, Petrides, Evans, & Meyer, 1993). Areas preparing saccades are involved in the cognitive aspect of saccade generation, and include the anterior cingulate gyrus (motivational modulation of saccades) and the dorsolateral prefrontal cortex ('dlPFC'; modulation of saccades based upon the environmental context; (Paus, Petrides, Evans, & Meyer, 1993; Petit et al., 1993).

The generated output signals are relayed, via the corticostriatal pathway, to the basal ganglia (discussed in further detail in the following section). From there, signals travel to the superior colliculus (SC) and brainstem, where motor impulses are generated and a saccade is initiated (see review by Sparks, 2002).

1.7.3.5 The role of the basal ganglia

The basal ganglia are aggregated subcortical grey-matter nuclei situated deep within the white matter. The basal ganglia influence human saccadic eye movements via two pathways: direct and indirect (elaborately reviewed by Hikosaka, Takikawa, & Kawagoe, 2000). Among the basal ganglia involved in saccadic regulation are the caudate nucleus (CN), external globus pallidus (GP_e), substantia nigra pars reticulata (SNr) and subthalamic nuclei (STN). The SNr, located in the midbrain, plays a particularly integral role as it receives signals from the CN (in the direct pathway), and the GP_e and STN (in the indirect pathway) as explained below.

As alluded to in the previous section, saccadic neural signals are relayed to the SC and brainstem before a motor impulse can be generated. The SNr tonically inhibits the SC, which leads to the suppression or termination of the saccadic neural signal (Hikosaka, Sakamoto, & Usui, 1989). For a saccade to be generated, the CN exerts a 'direct' inhibitory effect on the SNr, effectively dis-inhibiting the SC. This 'double negative' approach is thought to be more precise compared with a pure excitatory circuit (Hikosaka et al., 1989).

In the indirect pathway, the GP_e and STN (activated by GP_e and higher centres) stimulate the SNr—the function of which is to inhibit the SC—(Parent, Bouchard, & Smith, 1984). Therefore, this leads to the suppression of saccades, which may not always be undesirable. For example, fixating on a stationary object of interest requires the suppression of saccadic eye movements.

The two pathways, although appearing antagonistic, are not mutually exclusive, and often function in concert (K. Nakamura & Hikosaka, 2006). The net result is suppression of unwanted saccades whilst facilitating appropriate ones. It is when such physiological balance is disturbed that eye movement abnormalities arise. One

such ‘disturbance’ is the degeneration of the basal ganglia seen in PD (Yugeta et al., 2010).

1.7.4 History of eye movement recording

1.7.4.1 History of laboratory-based eye movement recording methods

It has been around 115 years since eye movements were first recorded; in 1901, Dodge and Cline produced the first photographic evidence of eye movement recordings (in Wade & Tatler, 2005). Since then, studies on human eye movement behaviour started appearing in the literature. Tasks during which eye movements were recorded were largely restricted to the confines of the laboratory. This was because the rudimentary eye-tracker systems utilised then had to be mounted on a bench, and for which recordings necessitated a stationary-held head. Eye movements were recorded from participants who were reading (Buswell, 1920), typing (Butsch, 1932) and viewing pictures (Buswell, 1935).

These early studies showed that ocular fixations precede motor actions. However, it was not until the seminal work of Russian psychologist Alfred Yarbus from the 1950s that an understanding of the role of human eye movements (especially saccades) in visual exploration was cemented (Yarbus, 1967). Instead of being mere reflexive responses, he showed that the behaviour of eye movements changes drastically based upon the task asked of the subject (i.e., a “top-down” control of eye movements).

1.7.4.2 History of dynamic eye movement recording methods

Bench-mounted eye trackers, although robust, restrained eye movement research to stationary tasks with a laboratory setting. To study the behaviour of eye movements during real-life dynamic activities, another eye tracking system was needed. In the

1950s, a British psychologist, Norman Mackworth, successfully devised a portable eye tracker which employed a helmet-mounted camera (Thomas, 1968). Although bulky and cumbersome to use, Mackworth and his colleagues were able to widen the circle of tasks for which eye movements could be recorded. In 1962, he was able to study the eye movements of drivers (Mackworth & Thomas, 1962).

Through the years, portable eye trackers decreased in size and increased in capability (Duchowski, 2003). Technology shifted from being based upon scleral shift coils to modern infrared video recording technology (van der Geest & Frens, 2002). By the 1990s, eye movement scientists finally managed to escape the laboratory and study eye movement behaviour in a range of real-life tasks, including: driving (Land & Lee, 1994), playing table tennis (Land & Furneaux, 1997), reading music (Furneaux & Land, 1999), making tea (Land, Mennie, & Rusted, 1999) and playing cricket (Land & McLeod, 2000).

Current portable eye trackers are much more sophisticated than Mackworth's (e.g., modern trackers utilise two cameras instead of one). Applications of remote eye tracking have expanded and the current technology has enabled a transition of the study of oculomotor behaviour from bench to bedside. Such clinical applications include the examination of eye movement patterns of radiologists (Kundel, Nodine, Conant, & Weinstein, 2007) and the study of gaze responses in visually-impaired children (Kooiker et al., 2016).

1.7.5 Eye movements in PD

1.7.5.1 Background

Given the crucial role of the basal ganglia in the generation of appropriate saccades, it is expected that abnormalities of saccades occur in basal ganglial disease (e.g., PD). In

fact, studies on saccadic parameters in PD have been reported since as early as the 1960s (Anderson & MacAskill, 2013). Until recently, however, results have been inconsistent and, if not contradictory (see examples in Crawford, Goodrich, Henderson, & Kennard, 1989; O'Sullivan et al., 2008; and Ventre, Zee, Papageorgiou, & Reich, 1992). Such disappointing results can be potentially explained by several factors as explained below.

Human development and ageing

Munoz and colleagues (1998) found saccade latency in humans decreases dramatically until around 12 years of age, and only starts to slowly increase past age 30. In another study, subjects over 50 years tended to perform memory-guided saccadic tasks less reliably and to be distracted more easily by visual stimuli (Fukuda & Hikosaka, 1994). Basal ganglial function was hypothesised to underlie such differences as the basal ganglia undergo development until the age of 12, and then start to undergo slow age-related degeneration after 50 years of age (Fukuda & Hikosaka, 1994). Because idiopathic PD is considered a “disease of the old”, age is less likely to have significantly contributed to such inconclusive results. However, it should still be taken into account in saccade studies involving young patients with familial PD and early-onset idiopathic PD.

Disease heterogeneity

Parkinsonism encompasses several heterogeneous, and often pathologically distinct, clinical syndromes. When “idiopathic” PD patients are selected for a saccade study, the diagnosis is usually made upon clinical criteria alone, without autopsy confirmation (see review in Brooks, 2012). Different parkinsonsian syndromes may have different saccadic profiles, leading to discrepancies if grouped together as a single sample in a study.

Effect of dopamine-replacement therapy

Another factor to consider when examining the eye movement literature in PD is the effect of dopamine-replacement therapy on saccades. Previous studies have revealed conflicting findings, including both improvement and deterioration of eye movement performance. Two mechanisms could explain such a discrepancy: task-specific effects of dopaminergic medications (e.g., reactive vs. memory-guided; Cubizolle, Damon-Perriere, Dupouy, Foubert-Samier, & Tison, 2014; Terao, Fukuda, Ugawa, & Hikosaka, 2013), and a form of dopamine-dysregulation dysfunction to the oculomotor system (Terao, 2014).

Methodological inconsistency

Most saccade studies in PD patients until around 2011 involved a small number of participants (Anderson & MacAskill, 2013). The resultant low statistical power can be another contributing factor to the reported conflicting results.

Cognition status

Cognitive status has been repeatedly shown to influence performance of saccadic tasks (Mosimann et al., 2005). PD patients displaying even mild impairment of cognition performed less well compared with PD patients with normal cognition (MacAskill et al., 2012). Failure to account for cognitive status may underscore the inconclusive findings of early saccades studies in PD.

1.7.5.2 Cognitive deficits in PD and eye movements

More recent saccades studies in PD, which have addressed some of the abovementioned factors, have started to shed light on particular patterns of saccade abnormalities in PD patients. Perhaps the most useful method of exploring the literature is by stratifying the studied PD populations according to cognitive status

for abnormalities of real-life (i.e., via clinical examination) and ‘artificial’ (i.e., in a laboratory setting) saccades.

Eye movement abnormalities in PD patients with normal cognition

It is thought that the manifestations of eye movement dysfunction in PD patients with normal cognition (PD-N) are directly due to the loss of dopaminergic terminals within the basal ganglia. Eye movement abnormalities that occur later in the course of the disease may instead be related a more widespread dopamine-independent neuronal loss (Terao et al., 2011). Clinical examination of eye movements may only reveal subtle abnormalities in patients with early PD, the most common of which are hypometric saccades (White, Saint-Cyr, Tomlinson, & Sharpe, 1983). In the laboratory, these patient exhibit hypometric memory-guided saccades and increased errors in antisaccades. Moreover, when asked to visually explore line drawings, PD patients were found to scan smaller regions (compared with controls), a result of smaller saccade amplitudes and longer fixation intervals (Matsumoto et al., 2011).

Eye movement abnormalities in PD patients with mild cognitive impairment

Clinical examination findings in PD patients with mild cognitive impairment (PD-MCI) are largely similar to those in PD-N patients. However, in the laboratory, there appears to be a direct correlation between cognitive dysfunction and saccade latency. Increased latency is particularly prominent in reflexive and memory-guided saccades (Terao et al., 2011). As cognitive status deteriorates, eye movement abnormalities similar to those observed in PD-D, though milder, start to manifest (MacAskill et al., 2012).

Eye movement abnormalities in PD patients with dementia

There is a paucity of published literature on eye movement dysfunction in demented PD patients (PD-D). In one study, PD-D patients were less likely to make predictive saccades and more likely to make errors when performing antisaccade tasks

(Mosimann et al., 2005). MacAskill and his colleagues (2012) found reflexive saccade latency, but not amplitude, to vary significantly with cognitive status, being more prolonged in PD-D patients than in PD-MCI patients (but not in PD-N patients compared with normal controls). Archibald *et al*, on the other hand, found PD-D patients to exhibit reduced saccadic amplitudes and longer fixation durations (2013). Their study, however, involved cognitively demanding visual exploration tasks, rather than random targets.

1.8 Neuropsychological testing

1.8.1 Introduction

Neuropsychological assessment began in the early 1900s, when educational psychologists Spearman, and later Binet and Simon, began trying to quantify the elusive notion of ‘intelligence’ (Boake, 2002). Since then, a plethora of tests have been developed, getting more refined and reliable by the day. The scope encompassed by such tests is vast, and what began as techniques to measure intelligence or screen army recruits have now been expanded to include innumerable educational tests. Such tests are helping scientists today in understanding the nature and varieties of mental abilities, both from normal and pathological perspectives.

This section provides an introduction to the neuropsychological tests used in my behavioural markers study. A brief history and description of the test is followed by a review of the test performance in different patient groups (especially PD). I then relate the test to the eye movements required to support it. I believe this will provide a unique insight into cognition and oculomotor control, especially in PD participants; such information is not ordinarily gathered when the tests are carried out in the traditional way. Lezak and her colleagues (2012; pp. 568) remark:

... numerical scores convey only a limited amount of information about an individual's performance. Careful observation of how patients proceed on constructional tasks and the types of errors they make is necessary to distinguish the possible contributions of perceptual deficits, spatial confusion, attentional impairments, organizational limitations, motor planning and/or execution difficulties, even motivational problems.

As examining eye movements would allow “careful observation” of how PD patients proceeded on neuropsychological tasks, this justified my motivation to study them. I decided to address the associations between eye movement and the performance on seven tasks (detailed below) in PD patients.

In the behavioural markers study, a battery of neuropsychological assessments was used to test key cognitive domains (discussed below), including executive function, language, learning and memory, processing speed, visuospatial function, and working memory and attention. Owing to the exploratory nature of the behavioural markers study, the main focus was to obtain an “overall impression” of eye movement behaviour during the performance of several neuro-psychological tests.

Standardised tasks were chosen for a number of reasons. First, the use of overly complicated tasks does not generally offer any additional advantage over simpler tasks. In fact, the opposite may be true; Lezak *et al* (2012) comment that complex tasks are generally less likely to yield meaningful information pertaining to a specific cognitive deficit. Furthermore, simpler tasks were easier to project visually, and the resultant eye-tracking data were not prohibitively laborious to analyse. Lastly, performing such neuropsychological tests while wearing an eye-tracker or being fixed to a machine is, at the very least, rather uncomfortable; relatively simpler tasks, with frequent breaks in between, provided the participants with sufficient comfort and ease to get through the whole study.

1.8.2 Cognitive domains

1.8.2.1 Background

Cognitive domains refer to the various mental processes that encompass a human's ability to acquire and understand knowledge. Naturally, such processes work in unison to allow for a smooth navigation of the world. Whilst a few neuropsychological tests have been designed as global assessments of cognition (e.g., the Mini-Mental State Examination), other tests attempt to examine the specific contribution of certain domains in a given task. This latter category of neuropsychological tests may be especially useful in trying to delineate the nature of "cognitive impairment" with which the patient presents. For PD patients, specific assessment of five cognitive domains has been recommended by the Movement Disorders Society Task Force (Litvan et al., 2011); these are: attention and working memory; executive function; language; memory; and visuospatial function.

1.8.2.2 Attention and working memory

This domain pertains to the ability to store, maintain and retrieve items on a short-term time-scale (Just & Carpenter, 1992). Neuropsychological tests used to assess attention and working memory including the digits forwards, backwards and sequencing tests, and the Symbol Digits Modalities Test (SDMT; discussed below).

1.8.2.3 Executive function

Executive function refers to the capability of simultaneously controlling and coordinating higher-order processes, such as working memory, task switching, planning and goal-oriented activities (Elliott, 2003). Executive function is assessed by

a number of neuropsychological tests, including the Category Switching Test, Stroop-Interference Test and Trail Making Test part B.

1.8.2.4 Language

Language involves a range of complex and intertwined skills (Saur et al., 2010). These include processes to express as well as to comprehend oral (i.e., speech) and written language. Such processes are the *sine qua non* of effective communication with others, and patients in whom language processes are defective (e.g., post-stroke aphasia) are particularly prone to mental health disorders especially depression (De Ryck et al., 2014). Two neuropsychological tests commonly used to examine language are the Brief Boston Naming Test and the Indiana University Token Test.

1.8.2.5 Memory

Memory refers to the ability to encode, store, consolidate and recall information pertinent to the long-term acquisition of knowledge about skills and experiences. Long-term memory has been categorised into implicit and explicit memories. Implicit memory is that which is subconsciously acquired and utilised; procedural memory, a form of implicit memory, refers to the ability to perform particular tasks without conscious awareness or attention to previous experiences (Schacter, 1987). Explicit memory (also referred to as declarative memory), on the other hand, involves the conscious recollection of concepts and personal experiences (episodic memory) as well as factual information (semantic memory; Ullman, 2004).

Of relevance to the behavioural markers study was the assessment of episodic memory of participants. This is because such memory is particularly affected by age-related as well as neuropathology-related cognitive impairment. In addition, episodic memory is relatively easier to assess than other types of long-term memory (e.g.,

procedural memory; Tromp, Dufour, Lithfous, Pebayle, & Despres, 2015). Relevant to the current thesis, recent research has examined the effect of anthocyanin powder, extracted from blueberries, on episodic-like memory in mice (Beracochea et al., 2016). In humans, episodic memory can be assessed both verbally (e.g., the California Verbal Learning Test-II) and non-verbally (e.g., the Brief Visuospatial Memory Test-Revised).

1.8.2.6 Visuospatial function

This domain comprises processes that not only enable a person to perceive the visual world (e.g., shapes, angles and details), but also perceive spatial relationship between objects and their environments and reproduce what they see if needed (Mervis, Robinson, & Pani, 1999). This intricate system allows for successful and meaningful navigation in the environment. Neuropsychological tests to assess visuospatial function include the Judgement of Line Orientation (JLO) and the Rey-Osterrieth Complex Figure copy test.

1.8.3 Neuropsychological tests used in the behavioural markers study

1.8.3.1 Judgement of line orientation

Judgement of line orientation (JLO) examines the visual perception of angular relationships. The participant is presented with two angle 'test' lines, in full or in part, and is asked to match them to 11 numbered 'reference' lines (which form a semi-circle below the test line pair; see examples in Figure 4-11). JLO exists in two forms, H and V, both of which possess the same items but arranged in a different order. A learning effect from re-testing subjects was inconsequential in normal controls and minimal in PD patients (Montse, Pere, Carme, Francesc, & Eduardo, 2001).

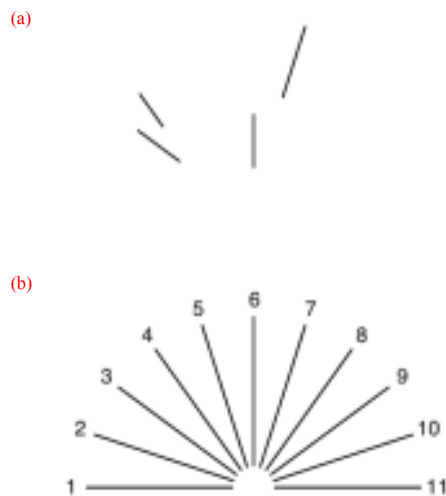


Figure 4-11. Two examples of JLO's test lines (a) to be matched to the corresponding reference lines (b); (Benton, Sivan, Hamsher, Varney, & Spreen, 1994).

The original JLO consists of 30 items, generally arranged in an ascending order of difficulty. Subjects are shown five practice items prior to commencing the actual test. Each test item is scored 1 for correct responses and 0 for incorrect responses; thus giving a score range of 0-30. Scores ≥ 23 are considered normal, while scores < 18 are considered a fail and often indicate severe cognitive deficit (Benton, Varney, & Hamsher, 1978). Scores of women and subjects older than 75 years tend to be consistently lower than scores of men and younger subjects. Therefore, score correction factors for age and sex are applied depending upon the participant's demographics.

PD patients have been reported to score significantly lower on the JLO test than matched normal controls. Performance does not appear to be influenced by disease severity or duration (B. E. Levin et al., 1991), although a single study did show a correlation with both parameters (Montse et al., 2001). PD patients were also found to score significantly lower on three short forms of the JLO (Gullett et al., 2013).

Visuospatial dysfunction has been proposed to underlie the inferior performance by PD patients (Hovestadt, de Jong, & Meerwaldt, 1987). Making such a claim can be problematic, however. This is because assessment of specifically visuospatial function in PD patients is often confounded by problems with attention, frontal lobe function, information processing speed and manual dexterity.

The nature of the JLO test means that it is unlikely to be confounded by frontal lobar dysfunction (Benton, Hannay, & Varney, 1975), or speed of processing or psychomotor abilities (Finton, Lucas, Graff-Radford, & Uitti, 1998). Nonetheless, the 'traditional' use of the JLO remains slightly challenging for PD populations. The reasons behind this are twofold; first, JLO does not differentiate an attentional deficit from a visuospatial dysfunction as an explanation for the inferior performance by PD subjects. Moreover, the 'traditional' method of scoring JLO (i.e., counting the number of correct and incorrect responses and assigning a single overall score) ignores potentially interesting information on the mechanism of errors made. To illustrate, the score of a subject who makes six systematic errors (solely right-sided items, for example) becomes indistinguishable from a subject whose six errors were spatially random.

A more detailed analysis by Ska *et al* (1990) classified JLO errors into different types (see Table 4-3). Using the Ska classification, analysis of errors made by PD subjects in one study revealed significantly higher rates of intraquadrant-type (QO₂ and QO₃; refer to Table 4-3) errors compared with normal controls and AD patients (Finton *et al.*, 1998). Another larger study corroborated these findings, as well as detecting horizontal-type (H) errors in PD patients (Montse *et al.*, 2001).

Table 4-3. Ska classification of JLO error types (1990; adapted with permission).

Abbreviation	Description
QO ₁	An oblique line is incorrectly identified as another oblique line and is different by only one spacing.
QO ₂	An oblique line is incorrectly identified as another oblique line and is different by two or three spacings.
QO ₃	Both oblique lines are displaced one or two spacings in the same direction, and maintain the initial spacing.
QO ₄	Both oblique lines are displaced without maintaining the initial spacing.
V	The vertical line is incorrectly identified as an oblique or horizontal line.
H	A horizontal line is incorrectly identified as an oblique or vertical line.
VH	The vertical line and one of the horizontal lines are incorrectly identified.
IQO	An oblique line is displaced from the original quadrant to the other quadrant.
IQOV	An oblique line is displaced from the original quadrant to the other quadrant, and the vertical line is incorrectly identified.
IQOH	An oblique line is displaced from the original quadrant to the other quadrant, and a horizontal line is incorrectly identified.

1.8.3.2 Montreal Cognitive Assessment

Introduction

The Montreal cognitive assessment (MoCA) was developed in order to overcome the limitations of the MMSE in detecting patients with MCI or early dementia (Nasreddine et al., 2005). Several reports indicated that the MMSE was inadequate in differentiating patients with MCI or mild dementia from healthy elderly individuals, primarily due to a ceiling effect (Ihl, Frolich, Dierks, Martin, & Maurer, 1992; Wind et al., 1997).

The MoCA is freely accessible online (www.mocatest.org), which contributes to its increasing popularity. It is available in three English versions to minimise any

learning effects, and is also available in many other languages and in a modified version for the blind.

The MoCA is an easy and quick tool to aid in assessing global cognitive status within the clinical setting. It takes 10 to 15 minutes to administer, and was designed to test eight different cognitive areas. These include: attention and concentration, calculations, conceptual thinking, executive function, language, memory, orientation and visuoconstructional skills. A 30-point scale assesses abstract thinking (i.e., commonality between two words), confrontational naming (i.e., three animal illustrations), digit repetition forwards and backwards, five-minute delayed recall of five words, orientation for place and time, sentence repetition, serial subtraction, sustained attention using target detection, verbal fluency for a single letter, and visuospatial and executive functioning (i.e., abbreviated trail making test, cube copying and clock drawing; Lezak et al., 2012; pp. 770-771).

MoCA in PD

In the original validation study (Nasreddine et al., 2005), a cutoff score of ≥ 26 was defined as normal. To account for the “education effect”, the developers proposed an addition of one point for subjects with 10-12 years of education, and two points for 4-9 years of education. Currently, the MoCA Normative Data for Age, Culture and Education trial is underway (Nasreddine, 2013); it aims to provide normative data across ages and education levels for 10 different languages and cultures.

To date, the MoCA has been successfully used in patients with various disorders including PD. The superiority of the MoCA over the MMSE in PD has been corroborated by multiple studies. The MMSE remains an unvalidated tool in PD, with no optimal screening cut-off score (Hoops et al., 2009), and inadequate sampling of executive functions (Zadikoff et al., 2008). Compared with the MMSE, the MoCA is less prone to a ceiling effect (Mamikonyan et al., 2009; Nazem et al., 2009), better

assesses cognitive domains particularly affected by PD (namely, executive and visuospatial functions; Gill, Freshman, Blender, & Ravina, 2008; Kasten, Bruggemann, Schmidt, & Klein, 2010), and holds adequate psychometric properties that support its use as a stand-alone cognitive screening tool (Chou et al., 2010; Watson et al., 2013). Furthermore, the MoCA demonstrated better diagnostic ability to delineate PD-N, PD-MCI and PD-D as compared with the MMSE. Suggested MoCA screening scores were PD-N ≥ 26 , PD-MCI 21-25 and PD-D ≤ 20 (Dalrymple-Alford et al., 2010).

Animal identification task

Confrontational naming refers to the ability to name objects when prompted by, in the case of the MoCA for example, line drawings. The process of naming is complex and commonly requires effective visual-perceptual, semantic as well as phonological processing (Tsang & Lee, 2003). Goodglass and Wingfield hypothesised a two-stage process to naming (1998). The first stage entails a direct link between an object and its name, leading to a quick response by the person. If this link is interrupted, however, a second stage is activated in which a slower associative process is involved. This way, the person might use associated words, concepts or sounds (e.g., rhinoceros for a hippopotamus) to self-prompt (Tartter, 1998).

Compared with their young counterparts, older subjects were found to make more errors and take longer when naming objects from pictures. These could be attributed to a multitude of reasons including age-related neuronal loss, as well as a reduction in the speed of sensory and motor processing (Tsang & Lee, 2003). Moreover, older subjects tend to be cautious and more rigid when performing cognitive tasks, generally leading to slower responses (Panek, 1978).

1.8.3.3 Matrix reasoning

The matrix reasoning (MR) task is made up of a series of visual pattern completion and analogy problems. The subject is required to choose the item that best completes the pattern from a multiple choice array. The test is presented in a 2x2 matrix (hence the name), and is arranged in order of increasing difficulty. See Figure 4-12 for an example.

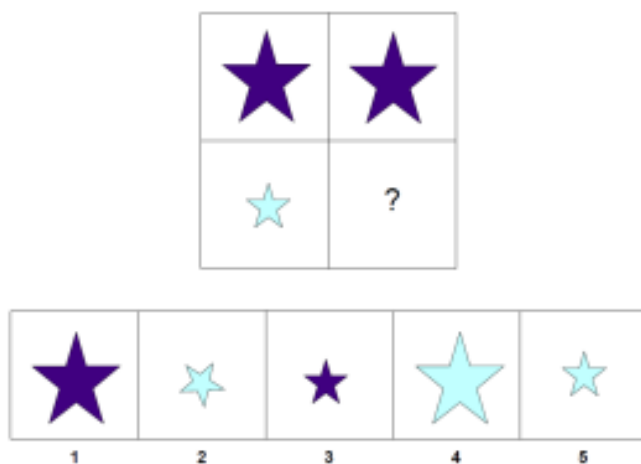


Figure 4-12. An example task of the MR (Harcourt Assessment Inc., 2005); reproduced with permission.

When MR was first introduced, it was designed as a measure of the subject's fluid intelligence (G_f). G_f is the ability to solve problems in novel situations, irrespective of prior acquired knowledge. G_f has been linked to the dorsolateral prefrontal cortex and anterior cingulate cortex (Duncan et al., 2000).

The MR task is thought to contrast with other WAIS-III tests, such as Vocabulary, which measure crystallised intelligence (G_c); this refers to the ability to solve problems based upon previous experiences and knowledge, and thus tends to

¹ The notion of a general intelligence (also known as the g factor) was first conceptualised by Charles Spearman, and later by his student, Raymond Cattell (Cattell, 1971).

improve with age. G_c has been linked to brain regions associated with long-term memory, including the hippocampus (Mangina & Sokolov, 2006).

The notion of MR measuring G_f has been recently disputed, however (Glascher et al., 2009). In a recent study by Travel *et al*, patient subgroups with prefrontal cortical damage (dorsolateral and/or ventromedial) did not score any differently on the MR task than patients with brain damage outside the prefrontal cortex (2009). In fact, scaled score means of the MR and Vocabulary tasks were identical for patients with prefrontal cortical damage. The authors explained that MR may actually be more of a “hold” test that reflects the subject’s premorbid intelligence (i.e., shows little change with brain damage).

To date, specific data on MR in PD patients are lacking. Almost all studies that have used the various versions of Wechsler’s scales in PD have failed to report the subscale data (i.e., individual test results, such as MR or Vocabulary results).

1.8.3.4 Visual search tasks (*Where’s Wally?*[™] and Jeddah map)

To be able to get by in daily life, humans are dependent upon intact “mechanisms of attention” (Lezak et al., 2012; pp. 426); that is, they ought to be able to direct their attention to a particular task, divide attention if distracted, and sustain attention until the task-at-hand is completed. Research on normal human behaviour when searching, maps for example, has been an area of active research. Colour-coding items on a map has been shown to reduce search times (Philips, 1979). Shive and Francis modelled map search times based upon target eccentricity and target-distractor colour distinctiveness (2013). Providing distractions (e.g., interactive maps), on the other hand, was disadvantageous to performance (Yeh & Wickens, 2001). Whilst research on eye movements on map search tasks *per se* is lacking, useful insights into human eye movement behaviour may be extrapolated from other visual search tasks.

Humans have been found to perform at a near optimal level during visual exploration (Chukoskie, Snider, Mozer, Krauzlis, & Sejnowski, 2013; Najemnik & Geisler, 2005). Despite exhibiting poor inter-fixation processing of information (Irwin, 1991) and not having perfect memory (Hayhoe, Bensinger, & Ballard, 1998), we are able to efficiently process information from each fixation. We achieve this by employing efficient eye movement strategies whilst utilising minimal neural resources (Najemnik & Geisler, 2005).

Visual search tasks vary in their complexity according to the required cognitive and attentional demands; they can range from simple free-viewing scenes to more demanding scenes (e.g., searching a map) or even specifically designed ‘puzzle’ scenes with busy backgrounds and one or more distractors (e.g., Where’s Wally?TM). Otero-Millan and his colleagues showed that saccade dynamics varied noticeably according to the scene shown and task(s) performed by participants (2008). Compared with a blank scene, participants made more saccades which were of shorter durations and smaller magnitudes when shown a Where’s Wally?TM scene (Otero-Millan et al., 2008).

1.8.3.5 Symbol digit modalities test

The Symbol digit modalities test (SDMT) is a symbol substitution test that predominantly examines speed of processing for material that demands complex and sustained attention. However, such skills as motor persistence, oculomotor scanning and visuomotor coordination are also required for good performance on the test (A. Smith, 1982).

In the reference key, each of the novel symbols is accompanied by a number from one to nine. Below the key are rows of boxes filled with any one of the symbols, below

each of which is an empty box where the corresponding number is to be filled in by the subject (see Figure 4-13). There is a total of 120 boxes (the first 10 are used for practice), and the subject is awarded one point for each correctly coded item. Age-stratified normative scores are available from the test manual (A. Smith, 1982).

≥	±	«	π	Ж	ψ	Δ	0	↑
1	2	3	4	5	6	7	8	9

ψ	±	π	ψ	±	0	≥	Δ	↑	Ж	±	«	±	≥	Δ
6	2	4												

Figure 4-13. The key and first row of empty boxes to be filled by participants completing the task (adapted SDMT version from Benedict et al., 2012).

Unlike other symbol substitution tests, the format of the SDMT offers three advantages. First, participants solve the test by writing numbers, a more familiar action when compared with drawing the symbols as done in WAIS-IV's Coding task. Moreover, the SDMT may be administered orally as well as in written format, as the responses require numbers rather than symbols. Third, because the symbols used contain three pairs of mirrored figures, the SDMT is potentially able to uncover more subtle difficulties with attention and orientation (A. Smith, 1982).

While the SDMT has been used in many neurological disorders, including AD (Fleisher et al., 2007), HD (Lemiere, Decruyenaere, Evers-Kiebooms, Vandenbussche, & Dom, 2002; Starkstein et al., 1988), MS (Huijbregts et al., 2004) and TBI (Draper & Ponsford, 2008), its use in PD has been less common. This is perhaps due to the fact that SDMT performance, especially for the written version, is speed-dependent; therefore, motor slowing in PD would be an evident disadvantage.

PD patients have been shown to perform worse on the SDMT compared with normal controls. In the studies published in PD groups, only the oral version of the SDMT was used. Hansch and his colleagues (1982) found a significant relationship between SDMT scores and P₃₀₀ latency. The P₃₀₀ is a class of long-latency positive components of event-related potentials that provide means of evaluating cognitive function independent of specific motor responses. Furthermore, Worringham *et al* have shown a significant correlation between driving safety and SDMT scores in PD patients (2006).

1.9 Overall aims and hypotheses

1.9.1 Overall aims

The overarching aim of the work presented (i.e., biological markers and behavioural markers studies) is to examine the role of different biomarkers at various stages of the disease process: early in the disease course when neuroprotectants are needed (i.e., blackcurrant anthocyanin and insulin-like growth factor-1 trials), in established PD (i.e., C-type natriuretic peptide trial) and as the disease progresses to involve cognition (i.e., exosomes trials and eye movement studies).

Specific aims are discussed in the introductory section of each chapter to follow. The hypotheses, based on the above review of the pertinent literature, are outlined in the concluding section below.

1.9.2 Project hypotheses

In the previous sections, I contended that a single biomarker is unlikely to encapsulate the multitude of clinicopathological aspects of PD. Therefore, I

hypothesised that several biomarkers of different categories and types ought to be employed at the various stages of the disease. This way, clinically meaningful changes in disease status—both motor and non-motor—may be captured.

I further hypothesised that analyses of metabolites present in biofluids— plasma and CSF—may potentially identify biological markers for assessing PD status and progression—these, in turn, could be employed in future clinical research, therapeutic trials, and overall patient care. Finally, I hypothesised that a detailed assessment of saccadic function during cognitively-demanding tasks in PD patients may assist in gaining greater insight into the effect of PD on behavioural aspects of oculomotor control.

CHAPTER 2

Methods of the Biological Markers Study

2.1 Participants

The protocol of this study was approved by the Upper South A Regional Ethics Committee, New Zealand (reference URA/10/03/022). The initial proposed sample size ($n = 20$) was opposed by the Ethics Committee who recommended “no more than 10 to 12” participants ought to undergo two LP for research purposes. All participants gave written informed consent before any study-related procedures were done. The study stretched over six months. The start date was 05/09/2010 and the stop date was 05/03/2011.

Participants were eligible to enrol in the study irrespective of the stage of disease or time since diagnosis. All participants were aged 40 years or older and met the Queen Square Brain Bank criteria for idiopathic PD as confirmed by a movement disorders neurologist (TA). All participants were recruited from the Van der Veer Movement Disorders Clinics and the New Zealand Brain Research Institute volunteer database, and were initially approached by the Primary Researcher (YA).

Exclusion criteria included any other explanation for parkinsonism (e.g., drug-induced parkinsonism) or a history of CNS disorders (apart from mild TBI). A history of a major medical illness which might impede on the absorption or excretion of blackcurrant antioxidant species, such as significant gastrointestinal or renal disorders, was also considered as an exclusion criterion. Likewise, participants with hypertension or metabolic disturbances (including protein synthesis disorders), or those who were smoking, excessively consuming alcohol (i.e., more than 15 standard units per week), pregnant, breast-feeding or obese (body mass index ≥ 30 kg/m²) were also excluded, since these factors have been reported to disturb normal functioning of the BBS (Haussermann et al., 2001; Robert, Godeau, Moati, & Miskulin, 1977). Finally, participants were excluded if they did not speak and read English, had major depression within the past six months, had a major cognitive or psychiatric disorder

or any other contraindications to study-related procedures, such as anticoagulation therapy or hypersensitivity to blackcurrants or gelatine.

2.2 Study setting

2.2.1 Study design

The study was designed as an open-label study of biofluids aiming at determining the concentrations of various biological metabolites in the plasma and CSF of PD participants. It was planned as a repeated measure, within-subjects design, comparing plasma and CSF metabolite concentrations four weeks apart. The study was divided into three visits, and all participants had to present to the NZBRI for these visits.

2.2.2 Visit one

This visit was to ensure that all participants had understood what the study involved and that they agreed to participate. An informed consent form was provided for all participants to sign; a photocopy of the form was supplied to the participant upon request. Next, the participants underwent several assessments of their PD course, as well as other general health and cognitive assessments. These included the Hospital Anxiety and Depression Scale (HADS), the MMSE, the Montreal Cognitive Assessment (MoCA) and the Movement Disorders Society (MDS) Unified Parkinson's Disease Rating Scale (UPDRS). The visit lasted about 75 minutes. At the end, participants were instructed to consume an anthocyanin-low diet starting 12 hours prior to each of their subsequent visits. Major constituents of this diet included white rice, white bread, chicken, tuna, white sugar, candies and non-fruit-based soft drinks.

2.2.3 Visit two

This visit took about 5 hours. It involved taking samples to measure metabolite baseline levels in the CSF (through an LP done by a clinician) and in the blood (through venepuncture of a vein in the antecubital fossa by the primary researcher). All patients were observed for a period of four hours in case post-LP headache developed. After that, participants were given the blackcurrant capsules and were instructed to take 6 capsules daily. This visit was designated day = 0 of treatment.

2.2.4 Visit three

This visit was four weeks after visit two (day = 28) of treatment and lasted about 6 hours. All assessments of PD, general health and cognition were repeated (i.e., LP, venepuncture, HADS, MMSE, MoCA and UPDRS). Participants were also encouraged to provide feedback on any improvements or adverse effects they had noted since starting the intervention. During this visit, participants took their last dose of blackcurrant capsules; and samples of CSF and blood were drawn again after 30-45 minutes of the last capsules consumed. All patients were observed for a period of four hours in case post-LP headache developed. This marked the termination of the blackcurrant anthocyanin treatment.

2.3 Cerebrospinal fluid sample collection and analysis

2.3.1 Setting

All participants were supplied with an information sheet on LP (Neurosciences Ward, 2005) to read while equipment was being prepared. Participants were encouraged to

ask questions. Risks were explained again verbally prior to the procedure. All LP were performed by a senior registrar-level clinician (neurology or anaesthesia).

2.3.2 Equipment

Cardinal Health® (Ohio, USA) lumbar puncture trays (see Figure 2-1) were used. We utilised 22G pencil-point (atraumatic) spinal needles with stylets. CSF samples were collected in coded plain Falcon tubes™ (BD Biosciences®, Massachusetts, USA). Powder-free latex sterile gloves (Nutex Dermshield, Ansell®, Australia) were worn at all times.



Figure 2-1. LP tray used in the biological markers study (reproduced with permission from Cardinal Health®, 2000).

2.3.3 Technique

All LP were performed with the participants in the lateral decubitus position. Most participants chose to lie on the left side, since it was easier given the setup of clinical rooms. Some participants, however, preferred to lie on the right side for one or both LP because of associated musculoskeletal symptoms (e.g., arthritis or back pain). Anatomical landmarks (i.e., anterior superior iliac crests for an L₃/L₄ level) were assessed by clinical examination.

An aseptic method was employed. The skin was cleaned with chlorhexidine gluconate 0.5% in 70% alcohol (Riotane, Perrigo®, Australia). Anaesthesia of the skin and soft tissues was achieved by injecting 5 ml of lidocaine 1% (Clarix, Multichem NZ Limited, NZ). Once in the subarachnoid space, CSF pressure was measured and recorded.

Around 7 ml of clear CSF was collected by each LP. One LP resulted in blood-contaminated CSF, which did not clear with continued dripping of CSF. The sample was discarded and another LP at a higher level (L₂/L₃) was attempted and resulted in obtaining clear CSF. The amount of CSF collected (around 7 ml) was substantially larger than the amount collected for clinical purposes (around 1-2 ml). Although seemingly high, greater volumes of CSF (as much as 30 ml) have been safely collected for research purposes in the past (Peskind et al., 2005).

2.3.4 Interim processing of samples

CSF samples were transported on wet ice to Endolab® (Christchurch, NZ) within 15 minutes of collection. Upon arrival, samples were taken to the cold room, where they were centrifuged at 3 000 revolutions per minute (RPM) for 15 minutes. The

supernatant was aliquoted equally between two plain tubes and stored under -80 C in alarmed freezers within 30 minutes of sample receipt.

Various analyses were conducted in several laboratories locally (i.e., CNP trial in 2011 and exosome trial in 2014), nationally (i.e., IGF-1 trial in 2015) and internationally (i.e., blackcurrant anthocyanin trial in 2011). Due to the different assay requirements of the various biological markers studied, the collected samples were used fresh, frozen and thawed as per the collaborators' recommendations. The CNP trial utilised fresh samples (i.e., pre-freezing). Frozen samples (one of the plain tubes from each participant) were sent internationally for the blackcurrant trial. Thawed samples were divided equally to allow sufficient volumes for the IGF-1 and exosome trials. The details of post-processing volumes of CSF samples sent to each laboratory are shown in Table 2-1.

Table 2-1. Volumes of CSF samples sent to each laboratory.

Table 2-1. Volumes of CSF samples sent to each laboratory.								
Sample status	Sample ID	BCA study	CNP study			Exosome study	Growth factor study	
		BCA	CNP/NTproCNP	cGMP	NEP	Exosomes	IGF-1	cGP
Laboratory site		Dundee	Christchurch			Christchurch	Auckland	
Before blackcurrant therapy	CAA9200	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9202	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9203	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9204	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9205	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9206	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9208	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9210	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9218	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9219	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9220	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
After blackcurrant therapy	CAA9201	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9211	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9212	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9213	1.6 ml	2.1 ml	1.1 ml	0.6 ml	0.6 ml	None	None
	CAA9214	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9215	Excluded as participant developed pneumonia around the time of the second visit						
	CAA9216	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9217	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9221	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9222	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
	CAA9223	1.4 ml	2.1 ml	1.1 ml	0.6 ml	0.5 ml	0.25 ml	0.05 ml
BCA = blackcurrant anthocyanins; cGMP = cyclic guanosine monophosphate; cGP = cyclic glycine-proline; CNP = C-type natriuretic peptide; IGF-1 = insulin-like growth factor-1; NEP = neprilysin; NTproCNP = aminoterminal fragment of proCNP.								

2.4 Plasma sample collection and analysis

2.4.1 Equipment

BD Vacutainer® Push Button Collection Sets (BD Biosciences®, Massachusetts, USA) were used. We utilised 21-guage needles. Blood samples were collected in coded EDTA- and heparin- coated BD Vacutainer® Push Button Collection Tubes (BD Biosciences®, Massachusetts, USA). Powder-free latex non-sterile examination gloves (Nutex Dermshield, Ansell®, Australia) were worn at all times.

2.4.2 Technique

All blood samples were obtained by venepunctures of the antecubital fossa on the asymptomatic side. All venepunctures were performed by the primary researcher (YA). For one participant, however, two attempts of obtaining blood samples failed, and he was taken to the Blood Service at Canterbury Health Laboratories (Christchurch, NZ) where blood samples were obtained after two further attempts.

An aseptic method was employed. The skin was cleaned with 70% isopropyl alcohol swab (Medi-Swab™, Smith & Nephew, London, UK) and allowed to dry. Around 15 ml (divided equally between EDTA and heparin tubes) were collected by each venepuncture. The amount of plasma extracted from the collected blood was approximately 4.5 ml.

2.4.3 Interim processing of samples

Blood samples were transported on wet ice to Endolab® (Christchurch, NZ) within 45 minutes of collection. Upon arrival, samples were taken to the cold room, where they were centrifuged at 3 000 RPM for 15 minutes. Plasma was aspirated into a plain tube

and stored under -80 C in alarmed freezers within 30 minutes of sample receipt. As with CSF samples, various analyses were conducted on the plasma samples in several laboratories locally (i.e., CNP trial in 2011 and exosome trial in 2014), nationally (i.e., IGF-1 trial in 2015) and internationally (i.e., blackcurrant anthocyanin trial in 2011).

2.5 Study endpoints

The primary efficacy endpoints were blood and CSF biological metabolite-concentrations four weeks apart. Secondary end points were changes seen in PD standard questionnaires (i.e., UPDRS, HADS, MMSE and MoCA) over the same time period.

The primary safety endpoints were reports of adverse events or incidental findings in CSF or blood analyses that would have negatively affected the results of the study. The participants were contacted by the primary researcher a day and again 10 days after their LP in case any adverse events had developed. Incidental findings of clinical significance by the CSF or blood analyses were directly discussed with the patient, by their physician (Prof. Tim Anderson).

2.6 Statistical analysis

Calculating the study power was a requirement of the application for ethical approval. With the initially proposed 20 participants, and a Type I error rate set at 0.05, a one-tailed repeated measures t-test would have had a power of 0.79 to detect an effect size of 0.5 or greater. Complying with the Ethics Committee recommendations for the sample size ($n = 11$) led to a decrease in power to 0.46. For

data that departed substantially from a normal distribution, nonparametric statistics (e.g., Wilcoxon matched-pairs test and Spearman's correlation) were used.

CHAPTER 3

Results of the Biological Markers Study

3.1 Study sample characteristics

3.1.1 Demographics

Eleven participants were enrolled in the biological markers study. The mean age was 66.5 years (± 11.0). All participants were male, and—except one participant who identified as Māori—all identified as Pākehā.

The mean duration since symptom onset was 6.1 years (± 2.5), while the mean duration since diagnosis was 4.2 years (± 2.6). At the time of the trial, all participants except one were on anti-PD medications (levodopa in nine, and monoamine oxidase inhibitor in one).

3.1.2 Anthropometric measurements

One participant developed aspiration pneumonia during the blackcurrant therapy period, and was unable to undergo the second LP. Data on his vital signs were, therefore, excluded from Table 3-1. Prior to the LP, participants' blood pressure and heart rate were recorded. Once the spinal needle was in the subarachnoid space, and before collecting the samples, CSF pressure was measured.

Table 3-1. Pre-LP vital signs for all participants except one.

		Visit 1	Visit 2	T-test	<i>p</i>
Systolic BP (mm Hg)	Mean	136	141	$t_9 = -0.53$	0.61
	SD	8	10		
Diastolic BP (mm Hg)	Mean	77	82	$t_9 = -1.22$	0.25
	SD	3	4		
HR (beats per minute)	Mean	75	68	$t_9 = 1.15$	0.28
	SD	5	3		
CSF pressure (cm H ₂ O)	Mean	15.3	13.8	$t_9 = 1.13$	0.29
	SD	1.9	1.4		
BP = blood pressure; HR = heart rate.					

Scores of the HADS were available for 10 participants, who completed the questionnaire both prior to commencing, and after finishing, the blackcurrant treatment. Participants scored significantly lower after receiving the blackcurrant therapy (see Table 3-2 for details). Significant reductions were present on the anxiety-subscale but not the depression-subscale scores when examined separately.

Table 3-2. Differences in HADS scores before and after blackcurrant therapy.

		Before	After	T-test	<i>p</i>
Total HADS	Mean score	9.5	5.4	$t_9 = 3.45$	0.007
	SD	6.8	3.9		
Anxiety sub-scale	Mean score	5.4	2.5	$t_9 = 2.69$	0.025
	SD	3.6	1.8		
Depression sub-scale	Mean score	4.1	2.9	$t_9 = 2.03$	0.074
	SD	3.5	2.7		

3.1.3 Clinical staging

Clinical staging of participants was performed using parts III and IV of the modified UPDRS (Goetz et al., 2007). Part IV of the assessment incorporates the modified Hoehn and Yahr scale (Goetz et al., 2004). Ten paired scores were available for both the UPDRS parts III and IV. No statistically significant differences were observed in motor function resulting from blackcurrant therapy (see Table 3-3).

Table 3-3. Mean scores of parts III and IV of the UPDRS before and after blackcurrant therapy.

		Before	After	T-test	<i>p</i>
UPRDS III	Mean score	34.5	31.6	$t_9 = 0.32$	0.32
	SD	12.5	11.7		
mH&Y*	Mean stage	2.2	1.9	$t_9 = 0.21$	0.21
	SD	0.2	0.7		

*** = modified Hoehn and Yahr stage**

The more-affected side was also assessed in participants. Data were available for nine participants only; almost half of whom had right-sided symptoms ($n = 4$), whilst the

other half had left-sided symptoms (n = 5). All participants reported they were right-handed.

3.1.4 Neuropsychological tests

There was virtually no change in the MMSE scores pre- and post- blackcurrant therapy. Moreover, even though the mean MoCA score after blackcurrant treatment was higher, this did not reach statistical significance. See Table 3-4 for details.

Table 3-4. Participant scores of neuropsychological tests and UPDRS pre- and post- blackcurrant treatment.

		Before	After	T-test	<i>p</i>
MMSE	Mean score	28.5	28.6	$t_9 = -0.21$	0.84
	SD	1.1	1.6		
	Range	27-30	26-30		
MoCA	Mean score	25.9	26.4	$t_9 = -0.89$	0.40
	SD	2.6	3.6		
	Range	22-29	21-30		

3.1.5 Discussion

3.1.5.1 HADS scores

Participants' HADS scores on the anxiety but not depression sub-scale significantly decreased (i.e., improved) after a month of receiving blackcurrant therapy. Several explanations can account for this finding (Alamri, MacAskill, & Anderson, 2016).

It could be suggested that the anxiety related to having an LP, especially for the first time, could have augmented HADS scores during the participants' first visit. Understandably, anxiety about such an invasive procedure is often experienced by both patients (Peskind et al., 2005) and their caregivers (Haimi-Cohen, Amir, Harel, Straussberg, & Varsano, 1996).

Studies to date on pre-LP anxiety have either utilised unvalidated anxiety measures (Peskind et al., 2005) or have investigated anxiety *after* the procedure has been completed (Haimi-Cohen et al., 1996; Peskind et al., 2005). In a study that evaluated anxiety before as well as after LP, scores were generally higher before the procedure (Nguyen, Nilsson, Hellstrom, & Bengtson, 2010).

What may argue against this explanation, however, is the lack of difference exhibited by participants on more objective measures. Physiological parameters (i.e., blood pressure and heart rate) were not different between the two visits. This makes ‘pre-LP anxiety’ less likely to have been a significant confounder.

It is difficult to attribute such positive effects to the blackcurrant treatment *per se* due to a number of reasons. First, a putative effect of blackcurrants may be biologically implausible in light of our findings of anthocyanins not reaching the CNS (see next section). Furthermore, participants of this open-label trial were pre-emptively aware of the intervention being administered, raising the possibility of a Hawthorne effect (e.g., altering their lifestyles or daily habits due to the knowledge of being observed). Finally, the attention our participants received during the study period could have positively affected their mood.

With such potential confounding difficulties, it may not be possible to assess whether the observed association could be true. High dietary berry-fruit intake has been associated with multiple health benefits (Devich, Pedersen, & Petrie, 2007), which could potentially include psychological wellbeing. Notwithstanding, the nature of the study cannot establish a true association between high blackcurrant anthocyanin intake and lower HADS scores in PD patients.

3.1.5.2 Clinical staging

Significant improvements on the UPDRS were not expected to be observed. PD is a chronic disease whose pathological processes are thought to start many years prior to diagnosis (Fearnley & Lees, 1991). The impetus for using neuroprotective agents in symptomatic PD patients is to slow or halt the neurodegenerative process, rather than restore lost dopaminergic cells (Yacoubian & Standaert, 2009). Still however, the biological markers study was not designed to examine this, as it was beyond the scope of the study.

3.1.5.3 Neuropsychological tests

There were no significant changes in the participants' scores of the MMSE or MoCA between visits. For the MMSE, scores did not improve despite administration of the same test, which indicates no significant learning effect. In anticipation of this potential problem when designing the study, it was decided that two different versions of the MoCA would be administered on the two visits. Therefore, any observed changes should be more likely to reflect true changes rather than improvement due to a learning effect.

One caveat is that the recruitment for this study did not control for the participant's baseline cognitive function. Most participants were thought to have normal cognitive function, although three participants demonstrated an element of mild cognitive impairment (i.e., low scores on the MMSE and MoCA). This was not formally evaluated at the time, however. Whether the observed 'no effect' of blackcurrant anthocyanins on cognitive status was due to a ceiling effect—a commonly cited problem for the MMSE—remains a speculation. A dedicated study of potential effects of blackcurrant consumption on cognitive status in PD patients with cognitive impairment may perhaps shed light on this.

3.2 *Blackcurrant anthocyanin trial*

3.2.1 Background and aim

Before human clinical trials on a new therapy could be started, areas of potential methodological pitfalls should be studied in detail to try to minimise them and to yield more definitive results. In the realm of dietary antioxidants as neuroprotective agents, these, according to Kieburtz, include: a sound scientific rationale, efficacy and relevance in animal studies, safety and tolerability and BBB penetration (2006). The first three of topics have already been covered quite extensively in the previous chapters.

There is no human study, to date, that has examined whether anthocyanins cross the BBB and be present in the CSF/brain in patients with neurodegenerative diseases. Therefore, the principle aim of this project is to examine whether any of the blackcurrant anthocyanin species cross the BBB and be present in the CSF of patients with PD. My hypothesis is that blackcurrant anthocyanin species would be detected in plasma, and also in CSF. A secondary aim of the study is to examine if any non-anthocyanin phytometabolites are present in human CSF and plasma after blackcurrant ingestion.

3.2.2 Methods

3.2.2.1 *Study intervention*

Blackcurrant anthocyanin concentrate capsules—Super Currantex® 20 (Just The Berries Ltd., New Zealand) were used in this study. Each capsule contained 100 mg of blackcurrant anthocyanins. Table 3-5 shows the nutritional facts of the trial capsules. Participants were instructed to take three capsules twice daily for a period of four

weeks, with the last dose taken at the beginning of Visit 3 (i.e., 45 minutes prior to biofluid sample collection).

Table 3-5. Nutritional facts of the blackcurrant capsules used in the trial (as supplied by Just The Berries, Ltd.); GMO = genetically modified organisms.

Main nutrients	Per 2 capsules (1.39g)
Energy	5.24 Kcal
Protein	204 mg
Fat	81 mg
Carbohydrate	874 mg
Vitamin C	200 mg
Main ingredients	Per 1 capsule
Currantex® 20 (NZ blackcurrant extract 120:1)	500 mg
Anthocyanins	100 mg
Other ingredient(s)	Gelatine capsule
Does not contain	Artificial flavours, preservatives, added sugar, salt, colours, gluten, wheat, yeast, dairy, soya, corn and GMO.

3.2.2.2 Pre-analysis treatment

Frozen CSF and plasma samples were sent to the James Hutton Institute, Dundee, Scotland. Samples were kept under -80 C at all times. Analysis of blueberries has not demonstrated significant effect on anthocyanin levels from sample freezing (Lohachoompol, Srzednicki, & Craske, 2004).

To ensure removal of proteins and possible contamination in order to increase sensitivity prior to analysis, samples were subjected to a clean-up process. Each sample was divided into five 200 µl aliquots. Duplicate samples were subjected to a universal extraction procedure developed at the James Hutton Institute into which 10 µg morin was added as an internal standard. This method (Dobson et al., 2004; Shepherd et al., 2007) uses a mixed chloroform, methanol, and water extraction stem

that yields two fractions, which contain polar (aqueous/methanol) and nonpolar (chloroform) metabolites (i.e., small molecular weight [< 2 kDa]). Briefly, samples were analysed on a LCQ-DECA system (Thermo, Hemel Hempstead, United Kingdom), comprised of a Surveyor autosampler, mass spectrometer-pump and photodiode array detector, and an iontrap mass spectrometer. Chromatography was performed on a C18 Synergi Hydro RP column ($4\ \mu\text{m}$, $2\ \text{mm} \times 150\ \text{mm}$; Phenomenex, Macclesfield, United Kingdom), and the mobile phase used for the separation of crude samples was a linear gradient spanning 35 min of 0.2% formic acid in deionised water to 0.2% formic acid in 90% acetonitrile at a flow rate of $200\ \mu\text{l}/\text{min}$. The sample injection volume was $10\ \mu\text{l}$, and the re-equilibration time was 10 min.

3.2.2.3 Chemical analysis

The method details have been described elsewhere (Fortalezas et al., 2010). The method employed has been shown to detect anthocyanin species in both *in vitro* (Fortalezas et al., 2010) and *in vivo* (Dobson et al., 2004; Shepherd et al., 2007) studies with high sensitivity.

In brief, metabolite analyses employed electrospray ionisation– mass spectrometer in positive- (proton charge) and negative- (electron charge) modes, using the following mass spectrometer conditions: sheath gas (N_2), 70 psi; auxiliary gas, 15 psi; spray voltage, 4 500 V; and capillary temp, 250 C. Only the polar fractions were analysed and three technical replicates were used. All measured metabolites (including non-anthocyanin compounds) were then identified using the Human Metabolome Database (<http://www.hmdb.ca/>).

3.2.2.4 Statistical analysis

Significance was defined as measurable difference in metabolite levels pre- and post-treatment observable in $\geq 50\%$ of patient population. A paired-samples t-test was conducted to compare anthocyanin levels before and after blackcurrant therapy.

3.2.3 Results

3.2.3.1 Subjective feedback

Most participants did not notice any symptomatic improvement, motor or otherwise. One participant, however, experienced substantial subjective improvement in his tremor, and continues to take blackcurrant supplements to date. The only adverse effect of the blackcurrant capsules, reported by several participants, was dark black stools.

3.2.3.2 CSF analyses

Number of samples

One participant developed pneumonia near the time of his second visit, and it was decided not proceed with the second LP. Therefore, a total of 20 samples (i.e., 10 paired samples) were sent for analysis.

Positive-mode analysis

A total of 23 metabolites were detected. None of the four main blackcurrant anthocyanins (C3G, C3R, D3G or D3R) were identified in any of the CSF samples. Moreover, none of the identified compounds (i.e., other polyphenols, endocrine molecules and drug metabolites) were significantly different between the pre- and post- blackcurrant therapy CSF specimens.

Negative-mode analysis

Forty-six metabolites were detected in total. Similar to positive mode analysis, no blackcurrant anthocyanins were identified, and the difference in concentrations of identified compounds did not reach statistical significance.

3.2.3.3 Plasma analyses

Number of samples

Blood samples were successfully collected from all participants. Extracted plasma samples were 22 in total (i.e., 11 paired samples).

Positive-mode analysis

Compounds identified in plasma were substantially more numerous, 75, compared with CSF. However, none of the blackcurrant anthocyanins were identified. Several of the identified compounds were significantly higher in the post-treatment samples compared with pre-treatment samples. See Table 3-6 for a list.

Table 3-6. List of compounds whose concentrations were significantly higher in plasma samples after blackcurrant therapy administration.

Compound	Mean pre-treatment response ratio	Mean post- treatment response ratio
Cortolone-3-glucuronide	0.77	1.22
Decanoylcarnitine	1.27	1.57
Epigallocatechin sulphate	0.85	2.48
Kaempferol	0.45	2.14
N-acetyl-L-glutamyl 5-phosphate	1.30	2.04
P-glycoprotein	0.84	3.27
UDP-L-rhamnose	0.59	2.19
<i>p</i> < 0.05; UDP = uridine diphosphate		

Negative-mode analysis

A total of 46 metabolites were detected. Similar to negative mode analysis of CSF, no blackcurrant anthocyanins were identified, and the difference in concentrations of identified compounds did not reach statistical significance.

3.2.4 Discussion

3.2.4.1 Subjective feedback

Objective extrapolation of findings from subjective reports of improvements or harm may be problematic at the best of times. This is perhaps because claims cannot be easily measured or validated, especially when reported *post hoc*.

It is interesting to note, however, that the participant who reported symptomatic relief of his tremor was the youngest (age 48 years). Whether this observation mounts to anything meaningful is doubtful given the small number of participants in the trial, and the known therapeutic benefit of the ‘placebo effect’ on PD tremor (Pogarell et al., 2002).

Stool colour may significantly be affected by the person’s diet. The dark pigment of anthocyanins is a well-established cause of dark/black stools in persons who consume large amounts of anthocyanin-rich diets (Escribano, Pedreño, García-Carmona, & Muñoz, 1998; Goehde, Ajaj, Lauenstein, Debatin, & Ladd, 2004). It is, therefore, not surprising that several participants reported this finding. The discoloured stool ought not to alarm the consumer; however, consumers of such supplements should be advised to seek prompt medical attention if faecal odour and consistency change—a worrisome sign of melaena (indicative of upper gastrointestinal bleeding).

3.2.4.2 CSF and plasma analyses

The blackcurrant anthocyanin trial did not demonstrate the presence of blackcurrant anthocyanins in CSF and plasma samples of PD patients after a month of oral blackcurrant concentrate supplementation. To the authors' knowledge, this is the first study to examine whether supplemental anthocyanins obtained through oral intake crossed the BBB in humans.

Our findings, while intriguing, were unexpected. The oral bioavailability of blackcurrant anthocyanins—as obtained from plasma of healthy subjects consuming blackcurrant smoothies—is known to be high, ranging between 91.7 and 96.5% (Kuntz et al., 2015). The mean time to maximal plasma concentrations varies between 58 to 68 minutes after ingestion of blackcurrant juice or smoothie (Kuntz et al., 2015). Whilst data on the anthocyanin bioavailability and time-to-maximal-concentration from blackcurrant capsule have not been formally studied, one would expect a reasonable rise in the plasma concentration after capsule ingestion—even to a just detectable level.

Given the results of previous research showing the presence of anthocyanins in blackberry-fed animals' brains, one would expect a similar presence in human brains. This apparent discrepancy, however, can be explained by several factors.

First, we analysed CSF samples as a proxy of the presence of anthocyanins within the CNS. While it is unlikely that dietary anthocyanins would be present in the brain without being in the CSF, we cannot rule out this possibility with certainty. Previous animal studies have used brain-tissue extracts instead of CSF samples; this approach is not justifiable in living human trials.

Second, animals used in previous studies were healthy animals, whereas our subjects had PD. Whether the compromised integrity of the BBB in PD patients affected the transport of anthocyanins to the CNS remains speculative. Current literature, however, argues against this possibility. For example, Kortekaas and colleagues have found an increased brain uptake of molecules that do not usually cross the BBB in PD rat models (2005). Moreover, BBB efflux transporters, such as P-glycoprotein, have demonstrated reduced activity in PD animal models (Schinkel et al., 1994) and patients on positron emission tomography scanning (Bartels et al., 2008). Therefore, it appears as if the BBB in PD can act like a safe that keeps molecules in and does not easily let them out.

Finally, the significance of the time-lag between last dose ingestion and sample analysis is unclear. Previous research has confirmed the presence of *intact* anthocyanins in healthy human urine, but not plasma, after consumption of blackcurrant juice (Hollands et al., 2008; Netzel, Strass, Janssen, Bitsch, & Bitsch, 2001). Jin *et al* were able to detect *breakdown metabolites* of blackcurrant anthocyanins in human plasma (2011). However, intervals at which blood samples were collected were not stated explicitly (“periodically up to 480 minutes after ingestion of [blackcurrant] juice”, Jin et al., 2011). In another study, anthocyanins were detected in plasma 1 (peak), 2 and 4 hours post-ingestion of purified anthocyanin supplements (Zhu et al., 2011).

3.2.4.3 Compounds that increased significantly in plasma post-treatment

Background

The significant increase in plasma levels of the following non-anthocyanin compounds after blackcurrant treatment was not necessarily expected. Of note, none of all identified compounds significantly *decreased* post-blackcurrant supplementation. The pertinent literature was examined in a *post hoc* attempt to

rationalise our findings. However, because the literature is relatively scarce, most of the assertions below are merely speculative.

Cortolone-3-glucuronide

Cortolone-3-glucuronide is an endocrine by-product that results from the hepatic glucuronidation of cortolone in the human steroid synthesis pathway (Hosoda, Yokohama, & Nambara, 1984). Levels of cortolone-3-glucuronide in biofluids have been assessed as potential biomarkers in the metabolic syndrome (Yu, Ning, Yu, & Tang, 2014) and in studies of traditional Chinese medicine (Li et al., 2015; Wang et al., 2015).

There are no studies that examine the relationship between cortolone-3-glucuronide and blackcurrant anthocyanins. However, in a study of eight individuals, urinary levels of cortolone-3-glucuronide significantly increased after consuming 400 ml of citrus juice (orange and lemon juice mixture) daily for four days (Medina et al., 2013). The authors concluded that the detected rise might have been due to upregulation of the pathway by one or more compounds found in citrus juice (Medina et al., 2013). Whether a similar mechanism accounts for the observed increase in our study remains a conjecture. Studies on cortolone-3-glucuronide in relation to blackcurrant consumption are lacking.

Decanoylcarnitine

Also known as acylcarnitine, this compound is involved in the regulation of fatty acid metabolism, ketone body synthesis and blood glucose homeostasis (McGarry & Foster, 1973; Williamson, Browning, Scholz, Kreisberg, & Fritz, 1968). Clinically, measuring blood levels of decanoylcarnitine has been found to be useful in differentiating the several biochemical phenotypes of the umbrella disorder, medium-chain acyl-CoA dehydrogenase deficiency (Gramer et al., 2015). To date, no studies have examined the relationship between decanoylcarnitine and blackcurrant

anthocyanins. Similar to the case of cartolone-3-glucuronide, blackcurrant treatment could have upregulated one or more pathways in which decanoylcarnitine is involved, leading to a demonstrable rise in plasma post-treatment.

Epigallocatechin sulphate

Epigallocatechin sulphate is the sulphated form epigallocatechin (EGC), a polyphenolic compound that belongs to the flavanol category of flavonoids (Kuhnau, 1976). EGC is most abundant in tea (especially green and, to a lesser extent, black varieties; Singh, Shankar, & Srivastava, 2011). Even when taken in mega-doses, EGC has poor oral bioavailability. However, it has been detected in both human plasma (Chow et al., 2003) and urine (Lee et al., 2002) after oral intake. No study to date has reported the detection of EGC after blackcurrant ingestion or its presence in human CSF.

Many health benefits have been attributed to EGC consumed in tea; perhaps more so than anthocyanins. Such benefits include anti-angiogenic effects (Domingo et al., 2010), anti-cancer properties (reviewed in Yang et al., 2011), anti-infective effects (Steinmann, Buer, Pietschmann, & Steinmann, 2013) and anti-inflammatory and antioxidant attributes in rheumatoid arthritis (Riegsecker, Wiczynski, Kaplan, & Ahmed, 2013). Additionally, a topical green tea-extract (containing EGC) has recently been approved for use in condylomata accuminata (Furst & Zundorf, 2014). There are several open trials under way investigating the use of EGC in many disorders, including AD (Friedemann, 2015) and Huntington's disease ('HD'; Priller, 2015).

Utilising the same rationale for using anthocyanins in PD (i.e., targeting OS), EGC has also been investigated in multiple *in vitro* and *in vivo* models of PD. EGC has been found to reduce the aggregation of α -synuclein monomers, thereby attenuating their toxicity (Lorenzen et al., 2014). Furthermore, EGC partially counteracted the effect of L-DOPA on cholinergic cell apoptosis (i.e., diminished dopamine autooxidation; Lee,

Choi, Lee, & Lee, 2013). In rat models, EGC reduced the neuroinflammatory response observed in lipopolysaccharide-induced PD (Al-Amri, Hagra, & Mohamed, 2013).

Human studies exploring EGC and PD have been less numerous. Epidemiological studies have demonstrated an association between tea (especially green) consumption and a lower risk of PD in Finland (Hu, Bidel, Jousilahti, Antikainen, & Tuomilehto, 2007), Singapore (Tan et al., 2008) and the US (Checkoway et al., 2002). In a phase II clinical trial, green tea polyphenol supplements resulted in significantly lower UPDRS scores at 6 but not 12 months (Chan, Qin, & Zheng, 2009). An ongoing clinical trial is investigating EGC in patients with multiple system atrophy (Levin, 2015).

It is apparent that it may be challenging, if not impossible, to disentangle which compound(s) directly lower PD risk. For example, the observed inverse association between PD risk and tea consumption (see review by Caruana & Vassallo, 2015) may have been due to caffeine and not EGC. On the other hand, EGC has also been found in *in vitro* studies of blackcurrant fruit (Butnariu, 2014) and leaves (Vagiri et al., 2015), although its level was the lowest compared with other polyphenols detected (Vagiri et al., 2015). Perhaps the observed lower risk of PD in populations who have high berry-fruit consumption (Gao, Cassidy, Schwarzschild, Rimm, & Ascherio, 2012) may have been due to a combination (or neither) of EGC and anthocyanins.

Kaempferol

Kaempferol is another phytochemical that is similar to EGC, but belongs to another flavonoid family, the flavonols (Kuhnau, 1976). Of the daily human intake of flavonols, kaempferol makes up around 20% (Liu, 2013), and is found in a variety of fruit and vegetables, including blackcurrants, blackberries, raspberries, peaches, broccoli, Brussels sprouts, cucumber, lettuce and squash (see review in Calderon-Montano, Burgos-Moron, Perez-Guerrero, & Lopez-Lazaro, 2011). In *in vitro* studies, kaempferol levels were found to be comparatively higher in the leaves (Vagiri et al.,

2015) than in the fruit (Mikkonen et al., 2001); no *in vivo* studies exist to date. Not only is kaempferol poorly absorbed (Barve et al., 2009), but it is also readily metabolised into its glucuronide, methyl and sulphate forms (Chen et al., 2010).

Kaempferol appears to possess a number of beneficial health properties; these have been enumerated in multiple pre-clinical studies: potent anti-inflammatory activities (Crespo et al., 2008; De Melo et al., 2009), antioxidant effects (Erben-Russ, Bors, & Saran, 1987) and cancer-deterrent properties (Lin, Juan, Shen, Hsu, & Chen, 2003). Clinical studies, on the other hand, have been much less common. In fact, there appears to be only one study that is currently under way that is examining the role of kaempferol (in ginkgo biloba) on intra-uterine growth-restricted infants (Abdellah, 2015). No studies have probed the relationship between kaempferol consumption and PD risk.

Other compounds

Several molecules were detected in significantly higher quantities in plasma post-blackcurrant treatment. These include: N-acetyl-L-glutamyl 5-phosphate, P-glycoprotein and UDP-L-rhamnose. The significance of these seemingly ubiquitous molecules is uncertain. No literature exists in relation to linking these compounds with blackcurrant intake or in PD patients.

3.2.4.4 Study limitations

The trial had several limitations that deserve to be mentioned. The sample size was small ($n = 11$), mainly as a result of complying with Ethics Committee recommendations. Regardless of the sample size, the study was mainly geared as a proof-of-concept trial; in fact, conducting a full clinical trial may not be feasible at this stage (i.e., a sample of at least 21 participants would be required to detect a large effect size with 80% power and Type I error rate of 0.05 or less).

Given the biological markers study only involved male participants, this limits the generalisability of the study results. Most studies on sex-based differences in the permeability of the BBB have been on animal models. In general, the BBB of female members of a species appears to be more permeable compared with males (see review by Oztaş, 1998), although this pattern was reversed in drosophila males when courtship factors were studied (Hoxha et al., 2013). The only indication of sex-specific differences in BBB permeability in humans comes from a single study in which females were found to have significantly higher CSF concentrations of endocrine amines compared with males (Odink, Korthals, & Vette, 1987).

This sex-based difference in BBB permeability, however, is unlikely to fully account for the negative findings of the blackcurrant anthocyanin trial. Gao and colleagues found berry-fruit intake was associated with a lower risk of developing PD, *more* so in men than in women (Gao et al., 2012).

The duration and dose of the blackcurrant therapy were chosen arbitrarily. However, the per-day intake of anthocyanins our patients received (600 mg per day) was well in excess of the reported average daily intake of 250 mg on a typical Western diet (Kuhnau, 1976).

Finally, analysis of CSF and plasma samples was conducted in another country. Every step in specimen collection and transport was meticulously planned and carried out in accordance with scientific protocols (see an example by Tworoger & Hankinson, 2006). However, while compromises to sample quality arising due to logistical complications are very unlikely, it remains a possibility.

3.2.4.5 Future research

The findings, if corroborated, could have implications in future study of the dynamics of human BBB in patients with PD, especially in regards to drug and molecular delivery. Whether the putative benefits of high berry-fruit intake in protecting against PD are due to the usually-associated healthy lifestyle (Prendergast, Mackay, & Schofield, 2016) or the berry-fruit flavonoid compounds themselves ought to be studied further. To conclude, blackcurrant anthocyanins are not detected in plasma or CSF of PD patients following therapy with high-dose blackcurrant anthocyanin concentrate.

3.3 *Insulin-like growth factor-1 trial*

3.3.1 Background

Endocrinology researchers took interest in IGF-1 and its metabolites probably well before any role of IGF-1 in neurological research was appreciated. With the recent surge in the incidence of acquired insulin resistance—most commonly in the form of type 2 diabetes mellitus—research into dietary habits has gained momentum (Goff & Duncan, 2010).

Among these studies have been assessments of the role of dietary fruit and vegetable intake on the levels of IGF-1 and its metabolites. For instance, higher intake of fruit and vegetables early in life was found to be associated with higher levels of IGFBP-2 (and perhaps IGFBP-3) in adulthood (Krupp et al., 2016). In a nematode model, an anthocyanin-rich feeding mixture was found to prolong the nematode's life-span. The authors proposed that the anthocyanins led to the activation of transcription factors

that, in turn, interfere with insulin/IGF-1 signalling pathways (Chen, Muller, Richling, & Wink, 2013).

The main aim of this study was to examine the effect of blackcurrant anthocyanin intake on CSF and plasma levels of IGF-1 and its metabolites in humans. This was done in an attempt to elicit a potential mechanism for the putative benefits on consuming berry-fruits in reducing PD risk; we hypothesise that this occurs through normalisation of the known IGF-1-resistance in PD.

3.3.2 Methods

3.3.2.1 Pre-analysis sample processing

CSF and plasma sample acquisition and pre-analysis treatment have been described in the previous chapter. Frozen samples were allowed to thaw in room temperature. Once thawed, half the volume from each tube was kept in Christchurch for the exosome trial (see following section), whilst the other half was shipped on wet ice via World Courier® (AmerisourceBergen, Pennsylvania, USA) to The Liggins Institute, University of Auckland (Auckland, New Zealand).

3.3.2.2 IGF-1 analysis

Plasma and CSF IGF-1 level were measured using a commercially available Human IGF-1 ELISA Kit (Crystal Chem Inc., Chicago, Illinois, USA). Eighty µl of antibody conjugate were added to each well of the 96-well microplate pre-coated with anti-IGF-1 antibody, then 20 µl of sample diluted 1: 21 in dilution buffer or 20 µl of standards were added to each well. The plate was placed on a shaker and incubated for an hour, then the well contents were washed five times using 300 µl of wash buffer per well. Subsequently, 100 µl of enzyme-conjugated anti-IGF-1 antibody were

added to each well and placed on a shaker to incubate for 30 minutes at room temperature, followed by another five times washing step using 300 µl of wash buffer per well. Then, 100 µl of substrate solution were added to each well and incubated for 15 minutes in the dark at room temperature, and the reaction was stopped by the addition of 100 µl of stop solution. The absorbance was measured using a plate reader at 530 nm excitation wavelength and 590 nm emission wavelength. The assays were repeated twice for CSF samples (due to the limited amounts of samples available) and four times for plasma samples.

3.3.2.3 cGP analysis

Sample extraction of cGP

cGP-d2 was used as an internal standard for cGP assay as described by (Singh-Mallah et al., 2016). cGP-d2 (50 µl of 500 ng/ml) was added to 100 µl of plasma, vortex mixed. The solution was transferred to a 1-ml Phree phospholipid removal cartridge (Phenomenex, Auckland, New Zealand) contained in a 4.5-ml tube. Five-hundred µl of 1% formic acid in Acetonitrile (MeCN) were added to the cartridge and centrifuged at 1 000 g for five minutes at 4 C to enable the collection of the filtrate. The filtrate was dried using a vacuum concentrator (1.5 millitorr 'mTorr' for an hour, then 0.7 mTorr for 45 minutes, at room temperature). The dried samples were reconstituted in 100 µl 10% methanol/water and transferred to a UPLC vial for quantitation then centrifuged at 500 rpm for five minutes at 4 C to sediment any remaining particulates. Standards were prepared by spiking cGP into charcoal stripped human plasma, quality control samples, with cGP at two different concentrations, were utilised and then subjected to the same extraction procedure as the samples.

HPLCms assay

The chromatography conditions consisted of a Synergy Hydro 2.5 µm column (Phenomenex) 100 × 2 mm with an initial mobile phase composition of 10%

methanol/90% water flowing at 200 µl per minute with a column temperature of 35 C. The mass spectrometry conditions consisted of electrospray ionisation in positive mode with a voltage of 4 000 V, a sheath gas flow of 30 ψ, an auxiliary gas flow of 2 ψ, and a capillary temperature of 250 C. Fragmentation was achieved with argon at 1.2 mTorr as the collision gas and a dissociation voltage of 35 V. The mass spectrometer was run in selective reaction monitoring mode with the following two transitions 155.1 to 70.2 m/z and 157.1 to 70.2 m/z utilised for cGP and cGP-d2 respectively. The retention time for both peaks was 3.6 minutes. Unknown samples were quantitated using the peak area ratio of cGP/cGP-d2 compared with the standard curve of known concentrations.

3.3.2.4 IGFBP-3 analysis

Plasma and CSF IGFBP-3 levels were measured using a commercially available Human IGFBP-3 ELISA Kit (Crystal Chem Inc., Chicago, Illinois, USA). Fifty µl of dilution buffer were added to each well of the 96-well microplate pre-coated with anti-IGFBP-3 antibody, then 50 µl of sample diluted 1: 505 in dilution buffer or 50 µl of standards were added to each well. The plate was placed on a shaker and incubated for an hour, then the well contents were washed five times using 300 µl of wash buffer per well. Subsequently, 100 µl of enzyme-conjugated anti-IGF-1 antibody were added to each well and placed on a shaker to incubate for an hour at room temperature, followed by another five times washing step using 300 µl of wash buffer per well. Then, 100 µl of substrate solution were added to each well and incubated for 30 minutes in the dark at room temperature, and the reaction was stopped by the addition of 100 µl of stop solution. The absorbance was measured using a plate reader at 530 nm excitation wavelength and 590 nm emission wavelength.

3.3.3 Results

3.3.3.1 Overview

Seven paired samples of CSF and 11 paired samples of plasma were available for analysis. See Table 3-7 for a summary of the results.

3.3.3.2 IGF-1

Neither CSF concentrations nor plasma concentrations of IGF-1 changed significantly after commencement of blackcurrant therapy. In addition, there was no correlation between IGF-1 concentrations in the CSF and plasma ($r = 0.09$, $p = 0.75$), with a CSF:plasma ratio of < 0.01 .

3.3.3.3 cGP

Concentrations of cGP in CSF increased significantly after blackcurrant therapy ($t_{1,6} = 4.58$, $p = 0.001$). Plasma concentrations, on the other hand, remained largely unchanged. The correlation between cGP concentrations in CSF and plasma was significant ($r = 0.69$, $p = 0.014$); however, there was no correlation between cGP concentrations before and after blackcurrant treatment in CSF ($r = 0.04$, $p = 0.87$) or plasma ($r = -0.12$, $p = 0.69$).

Blackcurrant supplementation had no effect on the cGP/IGF-1 ratio in either CSF or plasma. However, there was a significant correlation between plasma cGP/IGF-1 ratio and cGP levels in CSF ($r = 0.67$, $p = 0.01$).

3.3.3.4 IGFBPs

Levels of IGFBP-1 were generally low in plasma and undetectable in CSF; this was unchanged from before or after blackcurrant therapy. IGFBP-2 was the predominant IGFBP in plasma; blackcurrant anthocyanin intake did not alter its CSF or plasma concentrations, however. IGFBP-3, on the other hand, was the principle IGFBP in CSF, but its CSF and plasma concentrations were unaffected by blackcurrant therapy.

3.3.3.5 Correlation of biomarkers with patient characteristics

Plasma concentrations of IGF-1 were examined for any correlation with the patients' age, degree of motor impairment (as assessed by UPDRS-III) and degree of cognitive impairment (as assessed by the MoCA). No significant correlation was found between plasma IGF-1 concentration and age ($r = 0.1, p = 0.2$), UPDRS-III score ($r = -0.12, p = 0.96$) or MoCA score ($r = 0.22, p = 0.01$). Owing to the limited number of available CSF samples and the low concentration of IGF-1 detected, similar correlational analyses with patient characteristics could not be conducted.

3.3.4 Discussion

3.3.4.1 Overview

This preliminary study of IGF-1 and related molecules in PD patients revealed two significant findings. Concentrations of cGP in CSF are correlated with the cGP/IGF-1 ratio in plasma; this, therefore, suggests that changes in CSF concentrations of cGP may serve as an indirect biomarker for monitoring IGF-1 function in PD. Additionally, we found that blackcurrant supplementation is associated with a significant increase in the CSF concentrations of cGP.

3.3.4.2 IGF-1

IGF-1-resistance has been reported in PD, as evident by reports of, albeit inconsistent, raised IGF-1 concentrations in plasmas of PD patients (Numao, Suzuki, Miyamoto, Miyamoto, & Hirata, 2014; Picillo et al., 2013). Akin to hyperinsulinaemia observed in insulin-resistant diabetic patients (Weyer, Hanson, Tataranni, Bogardus, & Pratley, 2000), increased hepatic production of

IGF-1 may be a physiological response to IGF-1 resistance in PD patients; unlike the established relationship between hyperinsulinaemia and diabetes, however, the cause/role of IGF-1-resistance and PD is unclear.

The concentration of IGF-1 in CSF is approximately 1% of that in plasma, although no statistically significant correlation could be drawn between the two parameters. IGF-1 has been demonstrated to have a limited ability to cross the BBB (Thorne, Pronk, Padmanabhan, & Frey, 2004). This would support *de novo* synthesis within the CNS, although the less-likely speculation of slow uptake from peripherally circulating IGF-1 cannot be excluded with certainty.

3.3.4.3 cGP

Unlike IGF-1, cGP is a smaller and more lipophilic molecule (Guan et al., 2007); these properties allow easier crossing of the BBB. This likely explains the observed high concentration of cGP in CSF (around 52% of that in plasma pre-blackcurrant treatment and around 71% of that in plasma post-blackcurrant treatment). Another source of CSF cGP might be *de novo* metabolism of CSF IGF-1; this only appears to play a minor role, however (Yamamoto & Murphy, 1995).

3.3.4.4 cGP/IGF-1 ratio

We observed a statistically significant correlation between plasma cGP/IGF-1 ratio and cGP concentration in CSF. Because the majority of circulating IGF-1 is biologically inactive (Binoux, 1995), merely increasing total IGF-1 concentrations in plasma may not necessarily overcome the proposed state of resistance (within the limits of homeostasis). Instead, increasing cGP concentrations (a metabolite and also a regulator of IGF-1 bioavailability) may be another compensatory

mechanism for the proposed IGF-1-resistance in PD. This, in turn, may explain the observed high cGP/IGF-1 ratio.

Impairment of IGF-1 function has recently been reported in a small case series of obese women (Guan et al., 2015). The investigators demonstrated the utility of the plasma cGP/IGF-1 ratio as a biomarker for weight change. Whether this can be extrapolated to PD remains very theoretical. This is mainly because of the differences in pathophysiological parameters between the two conditions (see Table 3-8), although a recent review (Spielman, Little, & Klegeris, 2014) highlighted more links than previously considered in the literature.

Table 3-8. Major differences between IGF-1-related biomarkers in obesity and PD.

	Obesity	Parkinson's disease
Site of IGF-1 resistance	Peripheral	Central > peripheral
cGP/IGF-1 ratio	High in plasma	High in CSF > plasma
Main IGFBP	IGFBP-3 (in plasma)	IGFBP-2 (in CSF)
cGP = cyclic glycine-proline; CSF = cerebrospinal fluid; IGF-1 = insulin-like growth factor-1; IGFBP = insulin-like growth factor-binding protein.		

3.3.4.5 IGFBP

The profiles of the different IGFBP in PD are not well-documented. In a study of biofluid concentration of IGF-1-related biomarkers, PD patients had higher concentrations of all IGFBP compared with controls (Mashayekhi, Mirzajani, Naji, & Azari, 2010); the reported concentrations, however, were uniformly low. Moreover, Reijnders and colleague found IGFBP-2 to be the predominant IGFBP in the brain (2004) consistent with our findings.

3.3.4.6 Role of blackcurrant anthocyanins

The findings of the IGF-1 trial analyses lend some support to our conclusions derived from the present trial. We hypothesised a potential neuroprotective effect of blackcurrant anthocyanins on dopaminergic neurons, since no anthocyanin species *per se* could be detected in plasma or CSF. In this trial, we found cGP concentrations in CSF increased significantly after blackcurrant therapy. Dietary anthocyanins (from consumed berry-fruits) may exert their neuroprotective effect, if any, by influencing the BBB's endothelial/ependymal cellular milieu (leading to a subsequent change of molecular trafficking or vascular resistance, for example). Indeed, the effects of anthocyanins on improving endothelial function (Zhu et al., 2011) and vascular resistance (Liu, Li, Zhang, Sun, & Xia, 2014) have been demonstrated in *in vitro* and *in vivo* studies. Our conclusions, however, remain highly speculative given the small sample and the lack of larger more robust corroborative studies.

3.4 C-type natriuretic peptide trial

3.4.1 Background

In serum, levels of NTproCNP have been utilised as a marker for inflammation (a pathological process suggested to be a harbinger to PD development). Two studies by the same group have found elevated NTproCNP levels in sera of PD patients (Koziorowski, Tomasiuk, Szlufik, & Friedman, 2012), especially in patients receiving dopamine agonist therapy (Tomasiuk, Szlufik, Friedman, & Koziorowski, 2014). The authors concluded that drugs such as ropirinole had a potential pro-inflammatory effect. Neither study examined CSF levels of NTproCNP.

To date, there appears to be no study that has examined CNP levels in the CSF of PD patients. Given the paucity of CNP research in the CSF of PD patients, the main aim of this trial was to measure CNP (and related biomarkers) levels in collected biofluid samples. Our hypothesis is PD patients would have higher concentrations of CNP and/or NTproCNP in the CSF. A secondary aim was to attempt to draw any correlations between measured biomarker levels and disease characteristics of PD participants.

3.4.2 Methods

3.4.2.1 Pre-analysis sample processing

CSF and plasma sample acquisition and pre-analysis treatment have been described in the previous chapter. Sample analysis was undertaken in Endolab® (Christchurch, New Zealand).

3.4.2.2 Sample analysis

CNP assays utilised a commercial CNP-22 antiserum (Phoenix Pharmaceuticals, Belmont, CA). This antiserum also cross-reacts equally with CNP-53, which is the main bioactive form in the human CSF (Espiner, Dalrymple-Alford, Prickett, Alamri, & Anderson, 2014). Within and between assay coefficients of variation were 3.8% and 5.5% respectively at 9 pmol/l (Espiner et al., 2014). NTproCNP in CSF was measured by radio-immunoassay (as described by Schouten et al., 2011) after extraction using Sep-Pak® cartridges (Waters Corporation, Massachusetts, USA). Within and between coefficients of variation were 6.8% and 8.4% respectively at 14 pmol/l (Espiner et al., 2014).

3.4.2.3 Statistical analysis

Initial inspection of the data revealed multiple outliers which would have skewed relationships tested by the parametric Pearson's correlation. Therefore, nonparametric statistics were employed instead. In addition, results from the present study were compared with data from a population reference group (Schouten et al., 2011).

3.4.3 Results

3.4.3.1 CSF samples

A total of 21 CSF samples (11 pre- and 10 post- blackcurrant therapy) were available for CNP analysis. The median CSF concentration of CNP was 8.6 pmol/l (range 4.9-19.0), whereas the median NTproCNP concentration in CSF was 677 pmol/l (range 487-1340); see Figure 3-1. No correlations between CSF concentrations of CNP and NTproCNP were evident in samples collected from visit 1 ($r = 0.17$, $p = 0.61$) or visit 2 ($r = 0.26$, $p = 0.47$). The median NTproCNP/CNP ratio in CSF was 79.9 (range 53.7-144.4). Blackcurrant anthocyanin intake did not alter CSF concentration of CNP or NTproCNP.

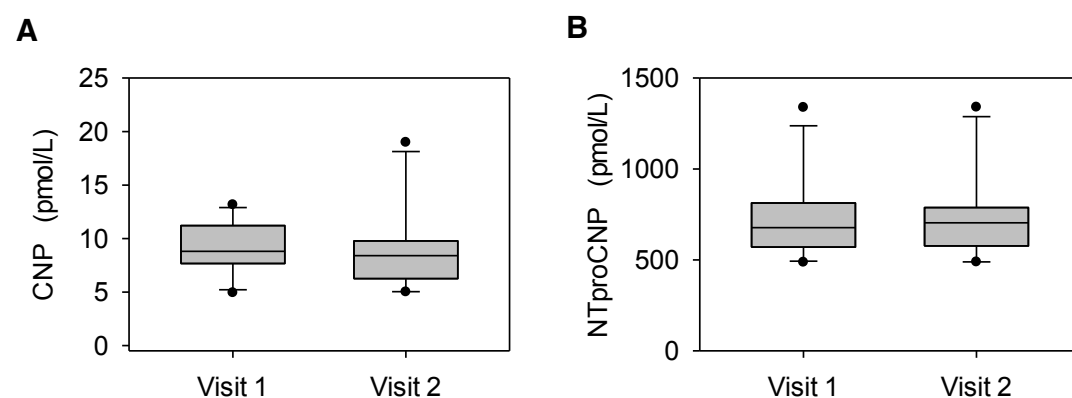


Figure 3-1. Median CSF concentrations of CNP (A) and NTproCNP (B) before (Visit 1) and after (Visit 2) blackcurrant therapy.

There was no significant association between CNP-related parameters in CSF and disease duration or severity. However, the NTproCNP/CNP ratio was positively associated with the participant's age ($r = 0.63, p < 0.001$). CSF opening pressure was negatively associated with age ($r = -0.61, p = 0.048$), but unrelated to NTproCNP/CNP ratios. No consistent effect of systolic, diastolic or pulse pressures was noted on the concentration of CNP or NTproCNP.

3.4.3.2 Plasma samples

A total of 22 plasma samples (11 pre- and 11 post- blackcurrant therapy) were available for CNP analysis. Whilst the median plasma concentration of CNP was 0.68 pmol/l (range 0.38-2.47), the median NTproCNP concentration in plasma was 17.7 pmol/l (range 11.8-33.2). The median NTproCNP/CNP ratio in plasma was 21.6 (range 11.8-46.6). Blackcurrant anthocyanin intake did not alter plasma concentration of CNP or NTproCNP.

Plasma NTproCNP and CSF NTproCNP concentrations were significantly positively correlated in visit 1 ($r = 0.86, p = 0.001$) but not visit 2 ($r = 0.59, p = 0.07$). Concentrations of CNP, on the other hand, showed no consistent correlation. There was no significant association between CNP-related parameters in plasma and disease duration or severity.

3.4.3.3 Outlier samples

All samples from one participant exhibited markedly high concentrations of CNP and NTproCNP on both visits, compared with the other PD participants. This particular participant was the oldest (age 80 years), and was on 14 different medications (i.e., polypharmacy due to other comorbidities). The CSF and

plasma ratios of NTproCNP/CNP were within the range of other PD participants, however.

3.4.3.4 Comparison with a reference group

Reference sample characteristics

The reference range of CNP-related values with which results from this study are compared came from a pool of 51 elective orthopaedic patients (18 male) who had undergone spinal anaesthesia prior to their procedures (Schouten et al., 2011). Only one patient in that sample had neurological disease (PD). The time of day for LP was similar in both samples. Likewise, most patient parameters, including co-morbidities, medications and vital signs, were similar—the only notable differences were the higher mean systolic blood pressure (mean 152 mm Hg vs. 128 mm Hg) and pulse pressure (mean 69 mm Hg vs. 47 mm Hg) in the reference group.

CSF analyses

CSF analyses from both samples are presented in Table 3-9. CNP-peptide concentrations in CSF do not appear to be affected by systemic haemodynamics or CSF pressure. However, samples from both groups exhibit a significant positive association between NTproCNP/CNP ratio in the CSF and age ($r = 0.77$, $p = 0.005$ in PD; and $r = 0.28$, $p = 0.045$ in the reference sample).

Table 3-9. CNP-related metabolites in CSF samples from the present study and a reference sample (Schouten et al., 2011).

	CNP trial	Reference group*	<i>p</i>
N	21	51	
NTproCNP (median, range)	677 pmol/l (592-782)	1 011 pmol/l (793-1 283)	0.006
NTproCNP/CNP ratio (median, range)	Visit 1: 80 (66-95) Visit 2: 71 (61-89)	130 (112-159)	< 0.001

*As reported by Schouten *et al* (2011).

Plasma analyses

In PD participants, a significant association between plasma CNP and NTproCNP levels was noted ($r = 0.61$, $p = 0.046$ for visit 1; and $r = 0.25$, $p = 0.47$ for visit 2). This close coupling of the two CNP forms was also found in the reference group (Schouten et al., 2011). Unlike the reference group, however, two contrasting patterns were observed in the PD participants: NTproCNP concentrations in plasma were strongly positively correlated with those in CSF in visit 1 ($r = 0.86$; $p < 0.001$) but not visit 2 ($r = 0.59$; $p = 0.07$)—whereas no relationship was found in the reference group (Schouten et al., 2011). Moreover, the significant inverse relationship between plasma and CSF levels of CNP found in the reference group (Schouten et al., 2011) was not observed in PD participants.

Plasma CNP concentration exhibited significant and positive association with the participant's age ($r = 0.83$, $p = 0.002$). However, plasma CNP was not found to correlate with systemic pulse pressure in either visit 1 ($r = 0.54$; $p = 0.09$) or visit 2 ($r = 0.15$, $p = 0.7$).

3.4.4 Discussion

3.4.4.1 General remarks

The present study is the first to report of CSF analyses of CNP metabolites in patients with PD. Our findings were largely in keeping with findings from a large concurrent study in which stored samples were obtained from the original DATATOP cohort (Espiner et al., 2014). In addition, samples from the present study were compared with values drawn from a reference age-matched population without neurological disorder (apart from subject with PD; Schouten

et al., 2011). Blackcurrant anthocyanins do not appear to affect the concentration of CNP or CNP in CSF or plasma.

3.4.4.2 CSF and plasma samples

In our participants, the concentration of NTproCNP in CSF was significantly lower than that of the reference group. Components of the CNP signalling pathway are found throughout the CNS (Herman, Dolgas, Rucker, & Langub, 1996), and hence, concentrations of CNP and NTproCNP are normally much higher in CSF than in plasma (Schouten et al., 2011). Because CNP undergoes rapid degradation and/or clearance by neural tissues, measurement of NTproCNP may be a more valid biomarker given its stability (Prickett, Yandle, Nicholls, Espiner, & Richards, 2001).

In PD, the activity of NEP-1—an enzyme that rapidly degrades CNP—has been shown to be reduced (Llorens-Cortes, Javoy-Agid, Agid, Taquet, & Schwartz, 1984). This may explain our findings of reduced NTproCNP/CNP ratio in the presence of unchanging CNP. In other words, there appears to be a concomitant reduction in both CNP production and clearance in PD (Espiner et al., 2014).

Plasma concentrations of NTproCNP in PD patients in the present study were thought to be lower than those of the reference group, although this was not statistically tested (E. Espiner, personal communication, May 27, 2014). Kozirowski *et al* (2012) reported raised plasma concentrations in patients with long-standing PD compared with controls. Reconciliation of this apparent contradiction in future studies is important—especially because a plasma-based biomarker is more practical (i.e., easier to obtain) than a CSF-based biomarker.

3.4.4.3 Other results

The differences in blood pressure parameters between our participants and those of the reference group cannot be explained, but are likely to be inconsequential to the CNP trial findings (Espinero et al., 2014). Whether pre-operative anxiety poses a significant contributor to the raised blood pressure observed is speculative, as the literature has not been consistent (Conceicao, Schonhorst, Conceicao, & Oliveira Filho, 2004; Lira et al., 2010). Due to the timing of sample analysis for CNP from the present study coinciding with the 2011 Christchurch Earthquake, CNP-related values were not tested or corrected for BNP cross-reactivity as was done for the reference range sample (Schouten et al., 2011). However, values of CNP peptides, in both CSF and plasma, replicated closely when re-measured at an interval of four weeks.

3.4.4.4 Future research

The comparability of the two study populations (i.e., PD participants and historical non-controlled healthy subjects) remains limited despite similar sample collection and analysis methods. However, several questions—both pertaining to CNP in general and specifically in PD—have arisen from our observations. The link between CSF NTproCNP and plasma NTproCNP observed in PD participants but not the reference group is intriguing; future efforts ought to corroborate such finding with concurrent control subjects, and aim to uncover any underlying pathophysiological mechanism(s).

The effects of medications on CNP are difficult to disentangle; all participants in both groups were using one or more medications (as high as 14 in the participant who had the highest CNP levels). In the case of PD, it would be pertinent to evaluate if one class of medications (e.g., dopamine agonists) affect CNP values

more than others. Inclusion of drug-naïve PD participants as well as drug-naïve controls may circumvent such potential confounding in future studies.

3.5 *Exosome trial*

3.5.1 Background

Extracellular vesicles in general, and exosomes in particular, have recently garnered increasing attention in the neuroscientific community due to their apparent role as propagation mediators of neurodegeneration (including PD; see recent review by Vella, Hill, & Cheng, 2016). This has, in turn, led to intensification of research efforts in the area. However, there have been concerns with the reliability and validity of methods used to isolate such extracellular vesicles (Vogel et al., 2011). The aim of this study, therefore, was to assess the repeatability of the tunable resistive pulse sensing (TRPS) method in human biofluid samples, as well as to examine the simple characteristics of exosomes in our PD participants.

3.5.2 Methods

3.5.2.1 *Pre-analysis sample processing*

CSF and plasma sample acquisition and pre-analysis treatment have been described in the previous Chapter. Sample analysis was undertaken by Izon Science, Ltd. (Christchurch, New Zealand).

3.5.2.2 Sample centrifugation

To remove any freeze-thaw aggregates, samples were spun (at 10 000 g for CSF and 3 000 g for plasma) for a duration of 10 minutes. The supernatant was then retained without disturbing the pellet. For CSF, 140 μ l of each sample was pipetted into microcentrifuge tubes to serve as raw reference samples; this left approximately 450 μ l of fluid to be purified as described below.

3.5.2.3 Sample purification

Biological samples tend to contain a mix of free proteins and other molecules that may cause fouling of equipment surfaces, as well as contaminating any subsequent analysis of vesicular content (Saugstad et al., 2014). Izon's proprietary separation column—the "qEV"—was utilised in order to remove such contaminants whilst retaining the majority of particles above 70 nm in diameter (Saugstad et al., 2014). Each sample was topped with 5 ml of phosphate buffered saline; the first 3 ml of the fluid was then discarded, leaving the "hot" fractions (i.e., containing exosomes) for subsequent analysis.

3.5.2.4 Quantification of exosomes

The present trial TRPS technology. In brief, a tunable sub-micron-sized pore separates two fluid cells: one containing the sample to be analysed, the other an electrolyte solution (Sowerby, Broom, & Petersen, 2007). An electric current between the cells is disrupted when a particle passes through the pore causing a translocation event, the magnitude of which is proportional to the volume of the particle traversing the pore (Roberts et al., 2010; Sowerby et al., 2007), and the blockade rate directly related with the particle concentration (Roberts et al., 2012). Calibration of the system with standard particles enables accurate

measurement of the concentration and size distribution of particles in solution (Roberts et al., 2012; Vogel et al., 2011). Often concentration measurement techniques only evaluate the 'total' particle concentration, which will crucially depend upon the smallest detected size of the technique used (van der Pol, Coumans, Gardiner, et al., 2014). In order to enable comparable exosome quantification, a particle concentration over a suitable size range is applied.

3.5.2.5 qNano system measurements

Samples were analysed on Izon's qNano systems using NP150- (plasma only) and NP200- (CSF and plasma) sized nanopores. Izon's EV reagent kit, which contains 200 nm calibration particles and a coating reagent to prevent non-specific binding of particles to the nanopore, was also utilised.

3.5.2.6 Further CSF sample processing

Raw CSF samples were diluted in PBS by 10x. A trial of ultracentrifugation (at 100 000 g) of a small volume of purified CSF samples was attempted in order to concentrate exosomes. This, however, resulted in tube damage and it was, therefore, not possible to obtain additional measurements of the CSF samples.

3.5.2.7 Further plasma sample processing

A concentration fraction, which refers to the concentration of particles in a sample always being quoted across a defined particle size-range, was utilised. This allowed for comparison of a set size-range between different samples. Due to time constraints, only half of the samples were measured under multiple pressures. Multi-pressure measurement is necessary to establish an accurate absolute concentration of particles (Vogel et al., 2011). See Table 3-10 for details.

Table 3-10. Summary of plasma sample-specific methods for exosome analysis.

	Multi-pressure measurements	Single pressure measurements
Participant samples	1, 2, 3, 4, 5 and 7	6, 8, 9, 10 and 11
Concentration fraction	90 – 300 nm	150 – 300 nm
Particle concentration	Absolute	Relative to the least concentrated sample

3.5.3 Results

3.5.3.1 CSF samples

A total of six CSF samples (i.e., paired samples, pre- and post- blackcurrant therapy, from three PD participants) were analysed. Both samples of one participant revealed slow particle rates, such that reliable measurements were precluded. Vesicle elution profiles revealed adequate purification process (B Glossop, personal communication, October 16, 2015). Findings from the other two participants are summarised in Table 3-11.

Table 3-11. Analysis of exosome diameter and concentrations in two PD participants.

		Participant 1	Participant 2
Diameter (nm)	Pre-BC treatment	Mean	132
		SD	52.6
		Mode	105
	Post-BC treatment	Mean	183
		SD	58.2
		Mode	135
Mean C₈₀₋₄₀₀ (particles/ml)	Pre-BC treatment	5.4 x10 ⁸	3.9 x10 ⁸
	Post-BC treatment	3.6 x10 ⁸	2.6 x10 ⁸
Mean C₈₀₋₁₈₀ (particles/ml)	Pre-BC treatment	4.4 x10 ⁸	3.2 x10 ⁸
	Post-BC treatment	2.0 x10 ⁸	2.1 x10 ⁸
BC = blackcurrant; C ₈₀₋₄₀₀ = concentration of exosomes between 80 and 400 nm (full size-range); C ₈₀₋₁₈₀ = concentration of exosomes between 80 and 180 nm (subset size-range).			

Given the insufficient data, statistical analyses were not carried. Analysis of most raw samples did not reveal significant discrepancies.

3.5.3.2 Plasma samples

Nineteen plasma samples (i.e., paired samples from nine PD participants and a single sample from the tenth participant) were available for analysis. Analysis of three samples, namely the paired samples of Participant 7 and the single sample of Participant 5, revealed only a small fraction of detectable particles at 90 nm. For these samples a larger nanopore was therefore used. Whereas this allowed for more accurate analysis of exosome size, cautious direct comparison with other samples analysed using the smaller nanopore is necessary. Results of multi-pressure analysis are shown in Table 3-12.

Table 3-12. Plasma multi-pressure analysis results pre- and post- blackcurrant treatment.

Nanopore	ID	C ₉₀₋₃₀₀ before BC-treatment (particles/ml)	C ₉₀₋₃₀₀ after BC-treatment (particles/ml)
Small	Participant 1	2.6 ×10 ¹⁰	1.6 ×10 ¹⁰
	Participant 2	4.4 ×10 ⁹	4.0 ×10 ⁹
	Participant 3	6.1 ×10 ¹⁰	7.3 ×10 ¹⁰
	Participant 4	8.5 ×10 ⁹	6.2 ×10 ⁹
Large	Participant 5	8.4 ×10 ⁹	
	Participant 7	8.3 ×10 ⁹	6.0 ×10 ⁹
BC = blackcurrant; C ₉₀₋₃₀₀ = concentration of exosomes between 90 and 300 nm; ID = identification number.			

Single-pressure analysis, which yields values relative to the least concentrated sample, can only give indicative concentrations. These are shown in Table 3-13.

Table 3-13. Single-pressure analysis results before and after blackcurrant treatment.

ID	C ₁₅₀₋₃₀₀ before BC-treatment	C ₁₅₀₋₃₀₀ after BC-treatment
Participant 8	1.0*	1.3
Participant 9	1.3	1.5
Participant 10	17.7	14.3
Participant 11	1.3	1.2

* = The least concentrated sample; BC = blackcurrant; C₁₅₀₋₃₀₀ = concentration of exosomes between 150 and 300 nm; ID = identification number.

3.5.4 Discussion

3.5.4.1 CSF samples

Characteristics (i.e., diameter and concentration) of exosomes found in purified CSF samples of the two PD participants were generally in keeping with those found in pooled CSF of 14 PD patients (Saugstad et al., 2014). A small peak of exosome concentration at around 200 nm was noted; this is likely to be a “carry-over” effect of the calibration particles from prior measurements. Using the C₈₀₋₁₈₀ (instead of C₈₀₋₄₀₀) has accordingly reduced the measured exosome concentrations in the present study.

Comparing the size profile of exosomes detected in the two PD participants with ovine CSF reveals a far broader range in the latter (Glossop, unpublished). The ovine CSF was extracted from brainstems of sheep shortly after death, which may explain the difference (e.g., presence of other vesicular particles).

Analysis of the CSF samples reveals two anomalies; exosome characteristics were unable to be collected from the third PD participant’s samples. This was due to slow particle rates, the reason of which remains unknown. Furthermore, the much higher concentrations of exosomes in Participant 2’s raw samples are

unexpected. A plausible explanation of this observation is that the thawed sample was not homogeneously mixed prior to centrifugation.

3.5.4.2 Plasma samples

Analysis of plasma content of exosomes failed to reveal any correlation between blackcurrant treatment and exosome characteristics; in some participants the measured concentrations marginally increased, and in others it slightly decreased.

Regardless of the effect of blackcurrant therapy, however, two patterns seem to emerge. First, the exosome concentrations for any single participant remain more or less stable. This is observed in spite of having samples being stored in different freezers, thawed and collected at different times and analyses performed on different nanopores using different techniques. Whether this is due to the short interval between collecting the two samples (i.e., 28 days between commencing and ceasing blackcurrant treatment) remains to be elucidated. The pattern, however, provides compelling evidence that a perturbation to sample collection, storage, preparation or analysis processes is unlikely.

The second observation is that some PD participants seem to have more exosomes in their plasma than others. To probe this observation further, clinical details of participants were re-examined *post hoc*. Participants with the highest plasma concentrations (namely, Participants 1, 3 and 10; see Figure 3-2) were found to score the lowest on MoCA (22, 24 and 22, respectively).

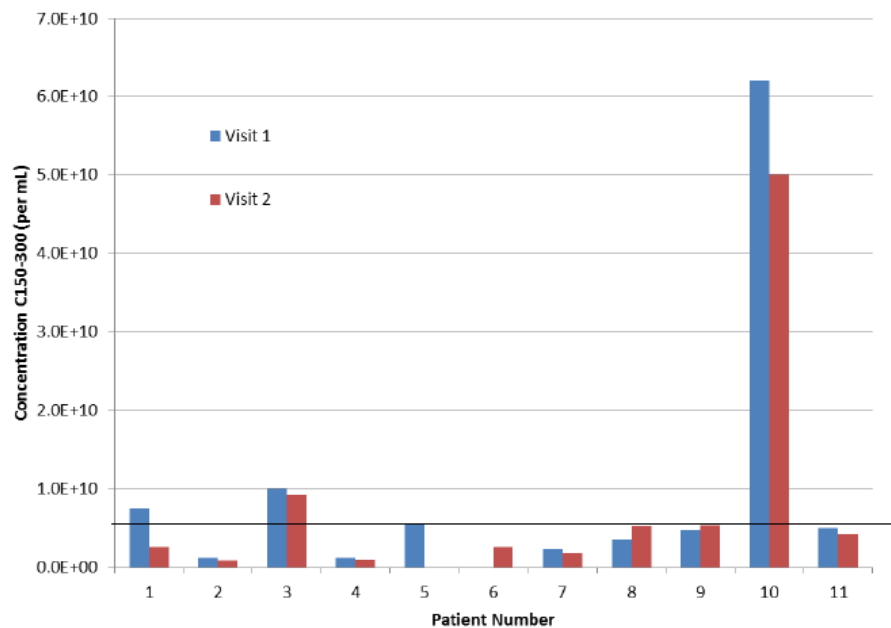


Figure 3-2. The relative concentrations of exosomes detected in 11 participants are shown, with samples from participants 1, 3 and 10 exhibiting the highest concentrations.

The observation we describe here is intriguing (Alamri, Vogel, MacAskill, & Anderson, 2016), but the study is insufficiently powered to draw definitive answers. Correlations between CSF exosome concentrations and cognitive impairment could not be examined since CSF samples were not obtained from all patients (including none of the three patients with the lowest MoCA scores). However, correlates using plasma, much more easily accessible than CSF, may be more appealing in clinical applications.

In a recent study by Stuendl *et al*, PD patients were have significantly elevated exosome concentrations in CSF compared with DLB and progressive supranuclear palsy (PSP) patients (2016). Unlike the Biological Markers study, the study by Stuendl and colleagues employed nanoparticle tracking analysis (NTA) to quantify exosomes, and the range of particle diameter was not specified. Likewise, plasma concentrations of exosomes were not measured.

The quantification of extracellular vesicles has presented several challenges despite the availability of several biophysical techniques, including NTA and TRPS (Izon Science, Ltd.). These challenges are mainly due to the small size and polydispersity of exosomes, and the lack of a standardised methodology for measuring exosomes which allows results from different days, users and instruments to be compared. The main advantages of TRPS over other technologies include its very limited dependence on instrument settings and its versatility in available measurement tools, such as the simultaneous determination of size, concentration and zeta potential of a given sample (Eldridge, Willmott, Anderson, & Vogel, 2014; Kozak et al., 2012; Kozak, Anderson, Vogel, & Trau, 2011; Vogel, Anderson, Eldridge, Glossop, & Willmott, 2012).

Characteristics of our sample and that of Stuenkel *et al.*'s (2016) are comparable (see Table 3-14). Perhaps most striking was the difference in the proportion of female PD patients. In the original report describing the Kassel cohort (Mollenhauer et al., 2011), almost two thirds of the patients were male—much more aligned with clinical experience in PD patients (Moisan et al., 2015). Regardless of this, however, no sex-based difference in exosome biological characteristics has been reported to date, and is therefore unlikely to significantly affect the results.

Table 3-14. Demographics of patients in the present study vs. the study by Stuendl and colleagues (2016).

Mean \pm standard deviation (range)	Exosome trial	Kassel cohort*
Numbers	10	37
Age (years)	66 \pm 11.5 (48-80)	73 \pm 7.9 (51-84)
Sex (% female)	0	70
Disease duration (months)	70 \pm 30 (11-122)	77 \pm 71 (10-300)
Hoehn and Yahr stage	2.2 \pm 0.2 (1-3)	3.9 \pm 1.2 (1-5)
MoCA score	25.6 \pm 2.6 (22-30)	Not performed
*As reported by Stuendl <i>et al</i> (2016); MoCA = Montreal Cognitive Assessment.		

In another recent study, Winston and colleagues examined the exosome profile in plasma samples of a group of participants with normal cognition, MCI (both stable and deteriorating) and AD (2016). Using NTA technology, exosome concentrations were not significantly different between the study groups. Analysis on exosome content, however, revealed significantly different levels of proteins in deteriorating MCI and AD participants, compared with participants with normal cognition or stable MCI (Winston *et al.*, 2016); some proteins were significantly higher in the former two groups (e.g., A β ₁₋₄₂), whereas others were significantly higher in the latter two groups (e.g., neurogranin).

Whether a similar pattern to our observation would be seen in CSF exosomes, and implications thereof, remains to be answered. Ridder *et al* have recently reported increased transfer of genetic material, via exosomes, between murine haematopoietic cells and Purkinje neurons in states of neuro-inflammation (Ridder *et al.*, 2014). This could afford a potential explanation for the higher plasma exosome concentration in PD patients with mild cognitive impairment (defined as MoCA score of 21-25 by Dalrymple-Alford *et al.*, 2010) compared with PD patients with normal cognition.

3.5.4.3 Other remarks

The initial results, whilst intriguing, raise more questions than they answer (see discussion below). This is because exosome research is still young, and standardised methods to isolate and identify these vesicles are still being developed and optimised (Colombo, Raposo, & Thery, 2014). Of particular relevance to the biological markers trial was the accurate determination of exosome size and concentration, and whether these variables provided meaningful, if any, clinical correlations. Tunable resistive pulse sensing, employed by Izon's qNano system, was recently found to be the most accurate (van der Pol, Coumans, Grootemaat, et al., 2014) and reproducible (Coumans et al., 2014) out of the five most commonly utilised methods to detect single extracellular vesicles.

A potentially problematic issue specifically pertaining to the exosome analysis was the 'freeze-thaw' effect and its ramifications on the quality of the samples. Immediately after the 2011 Christchurch earthquake, power was temporarily lost to the laboratory freezers; and even though back-up generators had restored power shortly thereafter, interval warming of frozen samples (estimated up to -60 C from -80 C, at most) cannot be excluded. This slight increase in temperature is unlikely to have caused significant damage to the frozen samples, even for species as temperamental as exosomes (Zhou et al., 2006). However, to err on the side of caution, only previously-unthawed samples that were placed in freezers least affected by the power cuts were used for exosome analysis; hence, the lower number of samples available. This, whilst preserving methodological rigour, resulted in limited data insufficient to draw any meaningful conclusions.

3.5.4.4 Future directions

Future research ought to focus on several aspects. Current protocols should be optimised further in order to allow for low-concentration-sample analysis. Vesicular particles below 80 nm could be investigated in future studies using smaller nanopores. Finally, the samples utilised in this study had been stored in the freezer for over three years; characterising exosomes in freshly collected CSF may offer different insights into the disease process, as well as establish the extent of the 'freeze-thaw' effect on exosomes in biofluids. Finally, larger studies that examine CSF and plasma concentrations of exosomes in PD patients stratified according to cognitive status are much needed.

CHAPTER 4

Methods of the Behavioural Markers Study

4.1 Participants

The protocol of this study was approved by the Upper South B Regional Ethics Committee, New Zealand (reference URB/11/06/010). Prior to the commencement of any study-related procedures, all participants gave written informed consent. This study stretched over a period of four years. The start date was 31/10/2011 and the stop date was 01/02/2015.

The study population was divided into three groups: PD-N group, PD-MCI group and a healthy control (NC) group. PD participants were aged 40 years or older, and met the UK Brain Bank criteria for idiopathic PD as confirmed by a movement disorders neurologist (TA). All PD patients were required to have had a normal brain scan (CT or MRI) within the previous five years, as well as having a normal neurological examination (apart from PD). Diagnostic criteria by Litvan *et al* (2012) were used to define mild cognitive impairment in PD-MCI participants. Control participants were matched for age (± 5 years), level of education (± 2 years) and sex. Years of education were calculated as the combination of years in primary school, years in secondary school (if any; see Table 4-1 for further details) and years in tertiary education (if any; see Table 4-2 for further details).

Table 4-1. Types of NZ secondary school qualifications and associated total years of education.

Secondary school qualification	Total years of education
No qualifications	10 years
School Certificate/NCEA	11 years
Sixth Form Certificate/University Entrance/ NCEA Level 2	12 years
Bursary/Scholarship/Higher School Certificate/NCEA Level 3	13 years
NCEA = National Certificate of Educational Achievement	

Table 4-2. Types of NZ tertiary qualifications and numbers of years added to the total after secondary school.

Tertiary qualification	Added years	Maximum total years of education
Trade Certificate	+1 or 2 years	15 years
Teaching or Nursing Diploma/Technician's Certificate/Undergraduate Certificate	+1 or 2 years	15 years
Polytechnic Bachelor's Degree	+3 years	16 years
University Diploma	+1 year	14 years
University Bachelor's Degree	+3 years	16 years
University Master's Degree	+5 years	18 years
University Doctorate Degree	+6 years	19 years

All participants were recruited from the Van der Veer Movement Disorders Clinic and the NZBRI volunteer database, and were initially approached by the primary researcher (YA). Exclusion criteria included any other explanation for parkinsonism, history of CNS disorders (except for mild TBI) and major cervical spine pathology. Patients were also excluded if they had major depression or a major psychiatric disorder, did not speak or read English or were younger than 40 years of age.

4.2 Study setting

4.2.1 Study design

The study was designed as a case-control study. The study involved a single visit to the NZBRI. The session lasted about two hours and was made up of three parts.

4.2.2 Part one

This entailed ensuring all participants had understood what the study involved, and that they agreed to participate. An informed consent form was provided for all

participants to sign; a photocopy of the form was supplied to the participant upon request. Information pertaining to ethnicity and previous medical history was directly sought from the participants. Next, the participants underwent several general health and cognitive assessments. These included HADS (Zigmond & Snaith, 1983), Edinburgh Handedness Inventory (Oldfield, 1971), UPDRS parts II, III and IV (for PD participants only; Goetz et al., 2007) and uncorrected visual acuity examination; a Snellen chart at 6 m was used for distant evaluation, and the N-notation chart (Collins, 1999) at arm's length (range from 30-35 cm) for near evaluation.

4.2.3 Part two

Laboratory-based saccadic function was then assessed in the Eye Movement Laboratory at NZBRI. The tasks presented, in order, were: animal identification task of the MoCA, JLO, MR, Where's Wally?TM and Jeddah map search. This part took about 60 minutes.

4.2.4 Part three

Participants were given a short break, and then taken to the MoVELab at NZBRI where real-life saccadic function was assessed. The tasks performed by participants, in order, were: SDMT₁, SDMT₂ and tea-making tasks. Most participants completed the tasks over a 45-minute period.

4.3 Equipment and software used in the study

4.3.1 iViewX™ Hi-Speed

The iViewX™ Hi-Speed system (SMI, Teltow, Germany) is a stationary non-invasive eye-tracker system. The manufacturer's specifications include accuracy of $0.25^\circ - 0.50^\circ$, monocular sampling rate of 1 250 Hz and tracking resolution of $< 0.01^\circ$ (SMI, 2010b). The device is composed of an eye-tracking column with adjustable ergonomic chin rest and forehead rest, a built-in infra-red eye camera and a see-through glass piece. The system is connected to a personal computer. Participants were seated with their chin on the chin rest and forehead against the forehead rest, while looking through the see-through glass piece at the presented stimulus. Stimuli were presented onto an 877 mm x 1 514 mm (width x height) screen via a DLP projector with a refresh rate of 100 Hz and a resolution of 1 280 x 720 pixels. The screen was 1 649 mm in front of the eye-tracking column. This system was used to obtain eye movement data in the Eye Movement Laboratory from lab-based tasks.

4.3.2 iViewX™ HED

The iViewX™ HED system (SMI, Teltow, Germany) is a mobile lightweight head-mounted eye-tracker system. This system was used to compile eye movement data in the MoVELab from real-life tasks. It is made up of two video cameras (eye camera and scene camera), both of which are mounted to a modified baseball cap (total weight < 80 g, SMI, 2010a) and connected by a USB cable to a laptop. This setup allowed for simultaneous recording of eye movements and the scene environment while allowing the participant to respond to tasks naturally. The manufacturer's specifications include accuracy of $0.50^\circ - 1.00^\circ$, sampling rate of 200 Hz and tracking resolution of $< 0.01^\circ$ monocularly (SMI, 2010a). Focal length of the scene camera was 3.6 millimetres, allowing for viewing angles of $\pm 31^\circ$ horizontally and $\pm 22^\circ$ vertically.

4.3.3 PsychoPy

PsychoPy is an open-source application for stimulus presentation and data collection (Peirce, 2007). It was used to command the beginning and end of each task, and control audio-response and video-clip recordings and stimulus target presentation.

4.3.4 BeGaze™

BeGaze™ is a behavioural and gaze analysis software produced by SensoMotor Instruments (SMI; Teltow, Germany). This eye-tracking software was utilised to extract eye movement data obtained by the iViewX™ Hi-Speed eye-tracker, as well as the iViewX™ HED system.

4.3.5 Audacity®

Audacity® (Mazzoni & Dannenberg, 2014) is free open-source software used for audio recording and editing. It was primarily used during analysis to play recorded voice responses as well as visualise the generated sound waves. Thus, a two-tier method was used to identify the start time of a voice response. A voice response was defined as the sound of the first letter of a word (e.g., the sound of the letter 'H' in *hippopotamus*) or stuttering of the first letter/syllable (e.g., *hip hip hip* hippopotamus). Use of indefinite articles (e.g., *a* hippopotamus) and conjunctions (e.g., hippopotamus *and* lion) were not considered proper voice responses based upon which start times ought to be documented. Start times were calculated twice by the same rater using the above method; this revealed an agreement of ± 10 ms.

4.4 Statistical analysis

Statistical analysis was performed using the SPSS Statistics® software package (SPSS Inc., 2013) and R software environment (Ihaka & Gentleman, 2015). Where reported, mean values and standard deviations (SD) are given as mean (\pm SD). Due to the expected performance progression of the three study groups, significant results from analyses of variance (ANOVA) were followed by a rather permissive *post-hoc* analysis, Fisher's least significant difference. Statistical significance was determined if Type I error rate was < 0.05 .

As the behavioural markers study was intended to be exploratory, numerous statistical tests were performed. As mentioned earlier, the emphasis was to obtain an in-depth evaluation of novel eye movement-performance associations; given the small sample size, an exploratory approach was taken. Therefore, corrections of family-wise error rates were not performed. To gain confidence in the results of individual findings, further research ought to be carried out in order to exclude the possibility of sampling variability accounting for the reported observations.

CHAPTER 5

Laboratory-based Eye Movement Tasks

5.1 *Introductory remarks*

5.1.1 Study aims and hypotheses

Exploring the relevant literature reveals an early interest in the interplay between manifest PD and disordered oculomotor control. This is evident by the early attempts of eye movement quantification in the 1960s (Anderson & MacAskill, 2013). A brief background on eye movement research in PD and the effects of the disease on oculomotor control has already been described earlier in this thesis. The aim was to better understand each group's patterns of performance and impairment. This was achieved by an in-depth evaluation of novel eye movement-performance associations.

Based on reviewing published literature, I hypothesised that cognitive and eye movement deficits are highly likely to correlate to the disease state—motor and cognitive impairment in particular—of PD patients. I further hypothesised that a detailed assessment of saccadic function with laboratory-based as well as more natural tasks may assist in gaining greater insight into effect of PD on the behavioural aspect of eye movement control. Because of the novelty of this approach, it is hoped to reveal potentially useful markers for PD, some of which may even prove easy to adapt for use in clinical settings as well as future studies.

5.1.2 Laboratory settings

5.1.2.1 *Settings in the Eye Movement Laboratory*

The ergonomic chin and forehead rests were cleaned with 70% isopropyl alcohol swabs (Medi-Swab™, Smith & Nephew, London, UK) prior to and at the end of each participant's session in the Eye Movement Laboratory. Comfort of seated

participants was ensured at all times, which occasionally necessitated placing a pillow between the participant's back and the chair's back-rest.

Recordings from the left eye (i.e., tracking the left pupil) were the default. Recordings from the right eye, however, were necessary for two participants in whom conditions, such as unilateral cataract surgery and trichiasis, precluded adequate recordings from the left eye. The iViewX™ Hi-Speed eye-tracker allows for recordings from either eye with no compromise of data quality (SMI, 2010). Participants were permitted to wear unifocal spectacles if desired; bifocal spectacles, however, interfered with proper tracking of the pupils, and participants needing to use them were excluded. The lights in the Eye Movement Laboratory were dimmed such that the only light was that projected onto the screen from the projector. The size and resolution of the objects depicted in the images were such that participants could perform all tasks comfortably.

5.1.2.2 Calibration protocol

After adequate positioning of the participant, and before starting tasks, a calibration procedure was undertaken. This comprised a semi-automated process in which the eye-tracker matched raw pupil and corneal reflection data from each participant relative to known fixation positions projected on the screen. Thirteen locations on the screen were pre-selected (see Figure 5-1); participants were asked to fixate on the randomly appearing calibration-targets, which were presented one-at-a-time.

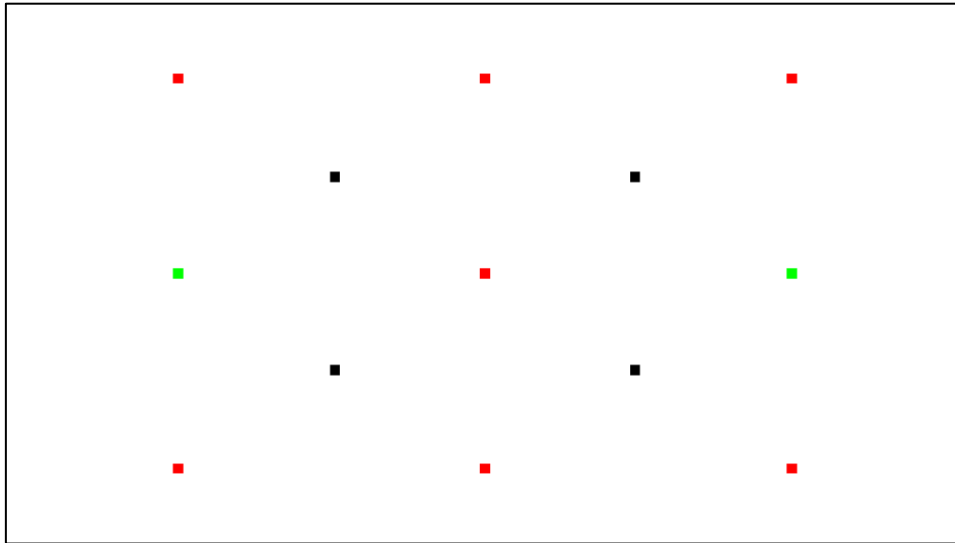


Figure 5-1. Calibration targets for the laboratory-based eye movement tasks.

The calibration-targets were distributed to give adequate coverage over an area marginally larger than the $\pm 15^\circ$ (horizontal) by $\pm 10^\circ$ (vertical) field in which stimuli were presented. These calibration-targets were set well below the maximum viewing angle of the iViewX™ Hi-Speed system, which is $\pm 30^\circ$ (horizontal), $+30^\circ$ (vertical upwards) and -45° (vertical downwards), (SML, 2010). The calibration process was repeated until satisfactory eye-position relative to the actual calibration-targets was obtained. Task-specific calibration checks were constantly reviewed between trials of the task (see below).

5.1.2.3 BeGaze™ analysis software settings

Automated data on saccadic eye movements were computed by BeGaze™ software (SML, 2013) offline. Relevant to this study were the saccade amplitude and the number of saccades made per task. Saccade amplitude was calculated by multiplying the mean saccade average velocity by saccade duration (SML, 2013). A task-specific upper limit of saccade amplitude was set to equal the maximum angle covered by that task's calibration check. The minimum saccade duration threshold was 22 ms. The saccade velocity detection threshold was $40^\circ /s$, and

commenced when 20% of a saccade length was attained and ended when 80% was reached. Automated data on fixations during task performance were also computed by the BeGaze™ software. Relevant to this study were the fixation duration and the number of fixations made per task. The minimum fixation duration was set at 50 ms.

5.1.3 Study sample

5.1.3.1 Demographics

Forty-eight participants were enrolled in the Behavioural Markers study; they were divided equally (n =16 per group) between the NC, PD-N and PD-MCI groups. The mean age of participants was 68 years (\pm 9.8), with a mean of 12.9 years (\pm 2.8) of education (see Table 5-1 for group-specific data). Most participants were male (NC = 88%, PD-N = 81% and PD-MCI = 81%). All participants identified as Pākehā, except one PD-MCI participant who identified as Māori. Three participants (one from each group) could not continue to be involved in the study beyond the initial meeting due to poor health or change of social circumstances; their demographic data have been included for completeness.

Table 5-1. Demographic information stratified according to group.

Group	N	Age		Education	
		Mean (year)	SD	Mean (year)	SD
NC	16	69.0	10.1	12.9	3.1
PD-N	16	66.2	9.8	13.4	2.9
PD-MCI	16	68.7	9.9	12.5	2.4
Total	48	68.0	9.8	12.9	2.8

Disease duration data (for PD participants) were available for all participants. Differences between both groups were not statistically significant (see Table 5-2 below for details).

Table 5-2. PD duration according to subjective report of symptoms and diagnosis by a clinician.

		PD-N	PD-MCI	T-test	<i>p</i>
Duration since symptom onset	Mean (years)	9.3	8.6	$t_{29} = -$	0.71
	SD	5.2	4.1	0.02	
Duration since diagnosis	Mean (years)	7.3	6.9	$t_{29} = -$	0.82
	SD	4.9	4.4	0.19	
Lag between symptom onset and diagnosis	Mean (years)	2.0	1.8	$t_{29} = -$	0.44
	SD	1.0	0.9	.83	

Disease durations calculated from the time of diagnosis were similar between the study's two PD groups (i.e., PD-N and PD-MCI). The reported rates of PD-MCI at time of diagnosis are 21.6% in the Norwegian ParkWest study (Pedersen, Larsen, Tysnes, & Alves, 2013), 35% in a study by Broeders *et al* (2013) and 42.5% in the ICICLE-PD study (Yarnall et al., 2014). The discrepancy in the observed rates is probably reflective of differences in the populations studied (clinic- vs. population- based) and the MDS-testing level diagnoses (level 1 vs. level 2 category diagnoses).

The lag between symptom onset and diagnosis in this study's sample is consistent with that reported in the literature (Cervantes Arriaga et al., 2014; Kraus, 1996; and see review by Meissner, 2012). Male sex and presenting with appendicular motor complaints (e.g., hand tremor; Breen, Evans, Farrell, Brayne, & Barker, 2013; Cervantes Arriaga et al., 2014) have been reported as independent risk factors for delayed presentation to a doctor. Female sex, on the other hand, is associated with delayed referral to a movement disorder specialist once a diagnosis is made or suspected (Saunders-Pullman, Wang, Stanley, & Bressman, 2011). This study, however, was not sufficiently powered to detect sex influences on the time of diagnosis or referral.

5.1.3.2 Anthropometric measurements

Utilising the Edinburgh Handedness Inventory (Oldfield, 1971), participant handedness was assessed. All participants were right-handed except three: two NC participants were left-handed and one PD-MCI participant was ambidextrous.

All participants had a distant corrected visual acuity (cVA) of 6/12 -2 or better in the better eye, with the exception of one participant whose distant cVA was 6/18 in the better eye. Moreover, all participants had a near cVA of N. 14 or better in the better eye, except one participant whose near cVA was N. 24 in the better eye.

Scores of the HADS were available from 45 participants. The mean scores ranged from 7.2 to 9.5. As a whole, participants with PD scored higher than NC participants, although not reaching statistical significance (see Table 5-3 for further information).

Table 5-3. HADS scores in the NC, PD-N and PD-MCI groups.

Group	N	Mean score	SD	ANOVA	<i>p</i>
NC	15	7.2	4.5	$F_{2,44} = 1.0$	0.38
PD-N	15	9.5	5.9		
PD-MCI	15	9.5	5.2		
Total	45	8.8	5.2		

5.1.3.3 Clinical staging

Clinical staging of participants with PD was performed using parts II, III and IV of the UPDRS (Goetz et al., 2007). Clinical assessments generally occurred mid-morning during which period all PD participants were in their “on” stage. Part IV of the assessment incorporates the modified Hoehn and Yahr scale (Goetz et al., 2004). No statistically significant differences were observed in motor function

between participants in PD-N and PD-MCI groups. Table 5-4 lists the mean scores of the various UPDRS components.

Table 5-4. Mean scores of the PD-N vs. PD-MCI participants on elements of the UPDRS.

		PD-N	PD-MCI	T-test	<i>p</i>
UPDRS II	N	15	14	$t_{28} = 1.3$	0.22
	Mean score	17.8	14.1		
	SD	8.3	7.1		
UPDRS III	N	15	15	$t_{29} = 0.34$	0.73
	Mean score	36.6	35.0		
	SD	13.1	12.4		
mH&Y*	N	15	15	$t_{29} = 0.58$	0.57
	Mean stage	2.2	2.0		
	SD	0.6	0.6		

* = modified Hoehn and Yahr

Which side was more affected was also assessed in PD participants; disease laterality was almost perfectly balanced between the right and left sides. In the PD-N group, more participants ($n = 8$) had right-sided symptoms compared with left-sided symptoms ($n = 7$). A similar trend was observed in PD-MCI participants where eight participants had right-sided symptoms, six had left-sided symptoms and one did not have this information recorded.

5.2 MoCA animal identification task

5.2.1 Background

PD patients have been shown to perform worse on confrontational naming tasks compared with matched controls (Hong et al., 2014). In PD, deficits in language have been described. Three aspects of language appear to be particularly affected in PD patients: comprehension, naming and verbal fluency (Bodis-Wollner & Jo, 2006). Impairments of comprehension and fluency have been attributed to

abnormal dynamics between the pre-frontal cortex and the basal ganglia (i.e., the “phonological loop”). Naming, on the other hand, appears to place less demand on executive processing (Bodis-Wollner & Jo, 2006). Tracking eye-movements while participants perform a naming task could potentially reveal (or confirm an absence of) any connection between naming and executive function (viewed as oculomotor planning in this case).

5.2.2 Methods

5.2.2.1 Instructions

The task instructions were explained verbally before the start of the task. Brief written instructions (“say the names of the following three animals”) were also projected onto the screen prior to the start of each trial. The three MoCA versions were always presented in the same order across participants. Participants were not specifically instructed as to which animal should be named first (i.e., all three animals were presented simultaneously, as on the paper form).

5.2.2.2 Definitions

The MoCA animal identification task was made up of three trials; each trial represented a MoCA version (see Figure 5-2). There were three side-by-side animals per trial (i.e., a total of nine animals to name). The first animal named by the participant was considered to be the first animal in that trial, regardless of its position on the screen (left, middle or right).

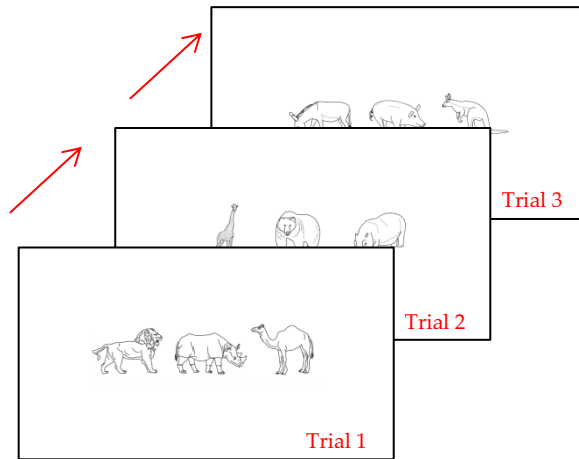


Figure 5-2. A visual representation of the order in which the trials were presented, corresponding to the three alternate forms of the MoCA test (Nasreddine et al., 2005).

5.2.2.3 Task-specific calibration check

Immediately prior to the presentation of each trial's stimulus, participants were asked to fixate on six pre-selected targets which were presented one-at-a-time. The targets were distributed to give adequate coverage over an area marginally larger than the field in which stimuli were presented (30° horizontally by 12° vertically). The last calibration-check target (referred to as the 'neutral point'), immediately prior to stimulus presentation, was located above the middle animal. The targets were presented quasi-randomly; the last target was always the (0°, 7°) target (i.e., the neutral point), while the first five targets appeared in random order. See Figure 5-3 below for an example.

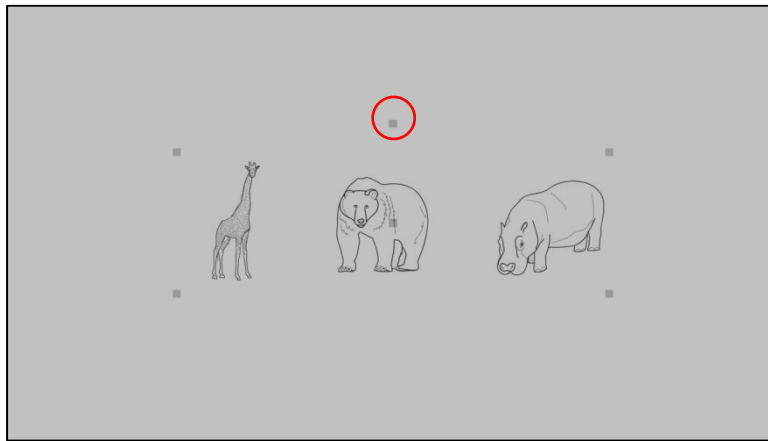


Figure 5-3. Animals of MoCA Trial 2 with overlaid calibration-check targets. The circled target was always the last to be presented (i.e., the neutral point).

5.2.2.4 Time to trial completion

The start of the trial occurred 250 ms after the onset of the neutral point. This consisted of the presentation of the stimulus and a simultaneous 400 ms A-note beep (which was used as a reference when recorded voice responses were played back in Audacity® for analysis). The trial ended when the researcher pressed any keyboard button after the participant uttered the name of the third animal. The total duration of the trial was measured, regardless of the need to prompt the participant at the start, hesitations, stuttering or incorrect responses.

5.2.2.5 Correctness

Participant responses were evaluated twice; notes were taken during task performance and recorded voice responses were evaluated once all data were collected. Acceptable responses, according to trial, were: (1) lion, rhinoceros and camel/dromedary; (2) giraffe, (polar) bear and hippopotamus; and (3) donkey/(jack)ass, pig/swine and kangaroo. Participants were allowed to self-correct. However, no hints were provided. Participants scored one point for correctly identifying an animal and zero points for incorrect responses.

5.2.2.6 Saccade and fixation data

An upper limit of 32.3° (maximum angular extent of the calibration area) was applied to saccade amplitude. This provided complete coverage of the area in which the MoCA task was presented. The number of fixations, rather than saccades, was chosen to be reported here as it was thought to be more reflective of the cognitive process of trying to identify the animal and recall its name (Martinez-Conde, Macknik, & Hubel, 2004). This is different to the methodology of Matsumoto *et al* (2011) who used the number of saccades when comparing visual scanning in PD patients.

5.2.2.7 Areas of fixation interest

Individual fixation locations were isolated using BeGaze™ (see Figure 5-4(a) for an example). Next, a rectangular background was specified as an area of interest (AOI) for each animal within a MoCA trial (see Figure 5-4(b)). Fixation points falling outside the AOI were excluded. Using Microsoft Paint, the “area of fixation interest” was depicted by connecting the outermost fixation points (see Figure 5-4(c)). Patterns in the areas of fixation interest were compared across trials between the three study groups. The method was adopted from (Matsumoto et al., 2011), although a quantitative scanning area was not calculated.

5.2.2.8 Latency to first animal fixation

This parameter refers to the time it took each participant to make a meaningful fixation (i.e., fixate on the first animal figure and not the surrounding white space), from the start of the trial. Because the starting location was the neutral point, this meant one or more saccades needed to be made before the eyes could fixate on any animal. Any interruption to eye-tracking (e.g., blinks or loss of tracking)

before the first meaningful fixation resulted in excluding that particular trial from analysis.

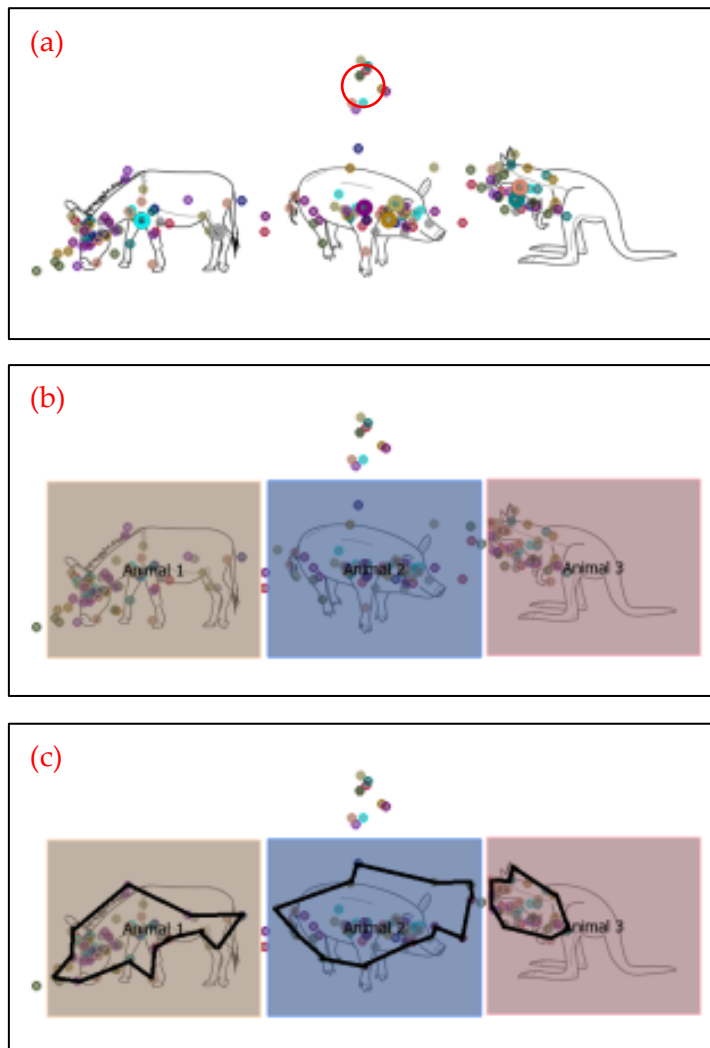


Figure 5-4. Step-wise illustration of creating areas of fixation interest around the MoCA animal stimuli. Individual fixation points, from all participants, were obtained (a). Rectangular areas of interest were specified for each animal (b). Areas of fixation interest were created by connecting the outermost fixation points (c). The red circle in panel (a) represents the neutral point location.

5.2.2.9 Latency to the first fixation per animal

This was the time taken to make the first fixation on *each* animal within a trial (i.e., including the second and third animals). For the first animal, this was as previously described (“latency to first animal fixation”). For the second and third animals, this was the time between the end of naming the previous animal and

beginning the fixation upon the next one. Any interruption to eye-tracking between animal fixations resulted in excluding that specific trial from analysis.

5.2.2.10 Latency to vocal response

This was defined as the time elapsed between the start of the first fixation on an animal and onset of the corresponding vocal response. A vocal response was defined as onset of the sound of the first letter of the animal's name (e.g., the sound of the letter 'H' in *hippopotamus*) or stuttering of the first letter/syllable (e.g., *hip hip hip* *hippopotamus*). Indefinite articles (e.g., *a* *hippopotamus*) and conjunctions (e.g., *hippopotamus and lion*) were not considered as the onset of a response. Responses for which the participant required prompting were excluded from analysis.

5.2.3 Results

5.2.3.1 Overview

Three participants (one PD-N and two PD-MCI) were unable to complete the confrontational naming-of-MoCA-animals task due to technical complications or physical exhaustion. Thus, 45 participants completed the task; 16 NC, 15 PD-N and 14 PD-MCI participants. Several parameters (see below) were recorded, each of which had associated quality-assurance criteria. Participants' data falling short of these criteria were discarded. Therefore, depending upon the parameter's criteria, the number of participants in an analysis was not always equal to the total number of participants completing the MoCA task.

5.2.3.2 Time to trial completion

The total time to complete each trial was recorded for every participant. Because participants completed each of the three MoCA animal identification trials separately, three durations (per participant) were recorded and then meaned. The per-group durations reported below represent the overall means of the group's means (see Table 5-5). There was no statistically significant difference between the three study groups.

Table 5-5. Time to trial completion according to group.

Group	N	Mean (s)	SD	ANOVA	<i>p</i>
NC	16	5.5	1.4	$F_{2,42} =$	0.51
PD-N	15	5.4	1.4		
PD-MCI	14	6.0	1.6		
Total	45	5.6	1.5		

5.2.3.3 Correctness

Most participants were able to correctly identify all nine animals: 98% for NC, 96% for PD-N and 94% for PD-MCI. Of the erroneous responses, confusing the rhinoceros for a hippopotamus (30%) and vice versa (25%) were the most common.

5.2.3.4 Saccade amplitude

Mean saccade amplitudes are presented in Table 5-6. NC participants made marginally bigger, albeit not statistically significant, saccades compared with PD participants.

Table 5-6. Saccade amplitudes across the study groups.

	N	Mean (°)	SD	ANOVA	<i>p</i>
NC	16	5.9	0.8	$F_{2,42} = 3.0$	0.061
PD-N	15	5.1	1.0		
PD-MCI	14	5.2	1.1		
Total	45	5.4	1.0		

5.2.3.5 Number of fixations and their duration

Although two trends emerged, in that NC participants made fewer, but longer, fixations and the opposite for PD-MCI participants, neither trend reached statistical significance. See Table 5-7 below for details.

Table 5-7. Fixation data revealed opposing trends, albeit non-significant, observed between the different study groups.

Group	N	Fixation number				Fixation duration			
		Mean (n)	SD	ANOVA	<i>p</i>	Mean (ms)	SD	ANOVA	<i>p</i>
NC	16	17.3	3.9	$F_{2,42} = 1.5$	0.23	237	55	$F_{2,42} =$	0.74
PD-N	15	18.1	4.4			223	54		
PD-MCI	14	20.6	7.6			220	81		
Total	45	18.6	5.5			227	63		

5.2.3.6 Areas of fixation interest

Visual inspection of the generated areas of fixation interest did not reveal any group-specific patterns (see Figure 5-5 for an example of MoCA trial 3). The head of the animal was the primary area of fixation interest. The notable exception was the giraffe, for which the primary area of fixation was the neck. It is notable that ‘less distinctive’ animals (e.g., donkey, pig and bear) had relatively larger associated areas of fixation interest, compared with ‘more distinctive’ animals (e.g., giraffe and kangaroo). This trend was evident in participants in all three groups.

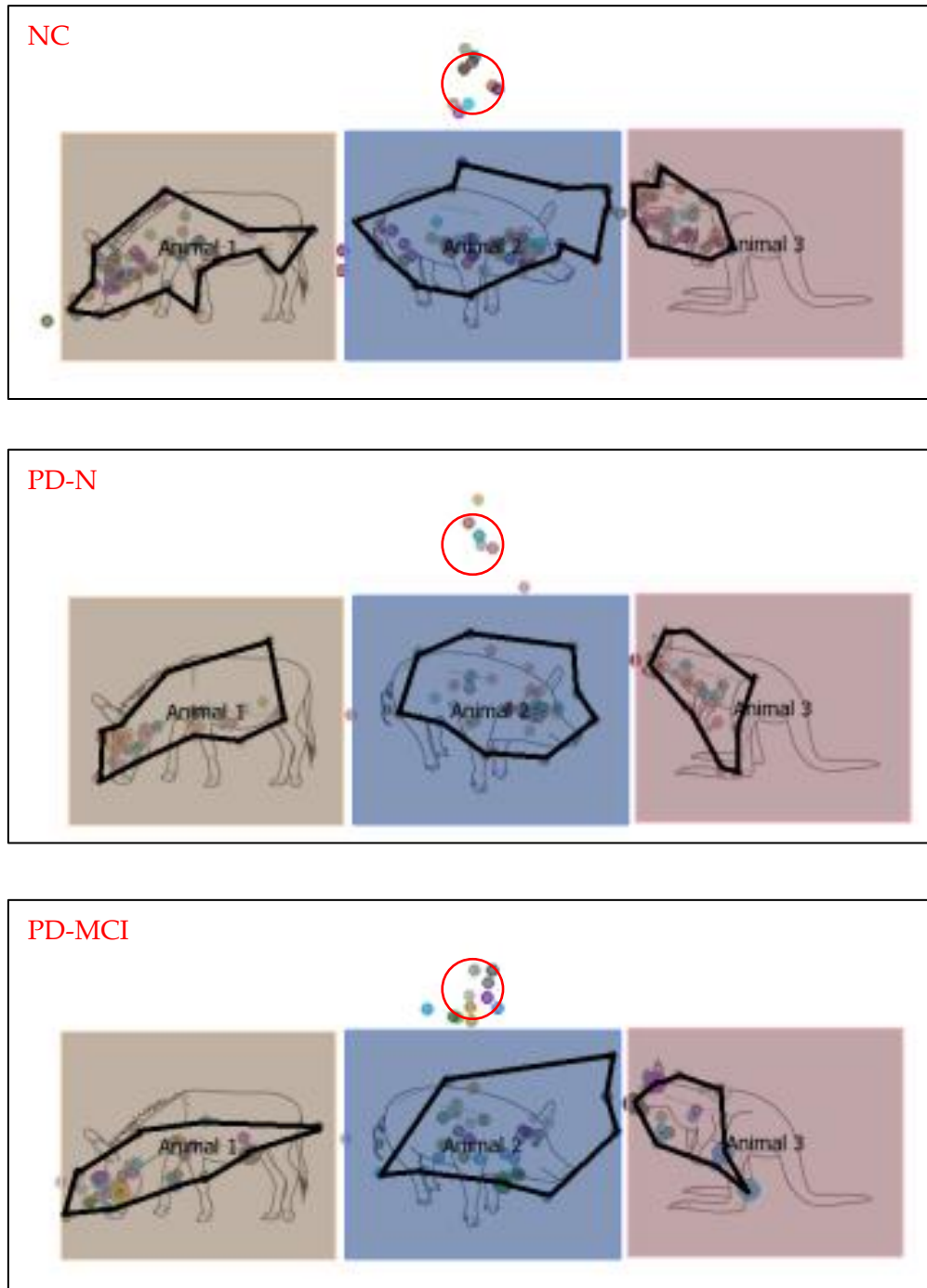


Figure 5-5. Patterns of the areas of fixation interest across groups for MoCA trial 3. Each participants' fixations, within a group, are given a different colour. The red circle represents the neutral point.

5.2.3.7 Latency to first animal fixation

Of a total of 135 individual recordings (i.e., recordings of each of the MoCA trials per participant), 15 were excluded due to unacceptable quality. The distribution of

excluded responses was almost equal between the groups: four from the NC group, five from the PD-N group and six from the PD-MCI group.

The times taken to fixate on the first animal of each MoCA trial were averaged. NC participants took a mean of 559 ms to fixate on the first animal, while participants in the PD-N group took 545 ms. PD-MCI participants, on the other hand, took the longest, with a mean of 727 ms. The difference did not reach statistical significance ($F_{2, 40} = 2.7$, $p = 0.08$).

To examine any learning effect as participants progressed through the task (i.e., first animal in MoCA trial 1 → first animal in MoCA trial 2 → first animal in MoCA trial 3), a linear mixed-effects model was applied (see Table 5-8 for details).

Table 5-8. Linear mixed-effects model fit by REML for latency to first animal fixation as a function of group. The mean latency for NC participants in trial 1 was utilised as the intercept (i.e., all subsequent latency values are relative to the intercept value).

	Latency (ms)	Standard error	df	t-value	<i>p</i>
NC trial 1	673*	204	70	3.29	
PD-N trial 1	-35	290	41	-0.12	0.90
PD-MCI trial 1	973	295	41	3.30	0.002
NC trial 2	-130	239	70	-0.55	0.59
PD-N trial 2	-2	339	70	-0.01	1.00
PD-MCI trial 2	-859	347	70	-2.48	0.016
NC trial 3	-196	231	70	-0.85	0.40
PD-N trial 3	39	334	70	0.12	0.91
PD-MCI trial 3	-488	350	70	-1.40	0.17
* = intercept; df = degrees of freedom.					

The average latency from trial start to fixating on the first animal in MoCA trial 1 was 673 ms for NC participants. Performance did not change significantly across MoCA trials 2 and 3. Performance of PD-N participants was not dissimilar to that of participants in the NC group. PD-MCI participants, on the other hand, were significantly slower in MoCA trial 1 (973 ms slower than NC participants, $p = 0.0020$). They, however, showed evidence of a learning effect between MoCA trials

1 and 2 ($p = 0.016$), but no significant change thereafter. See Figure 5-6 for the trends of per-group performance.

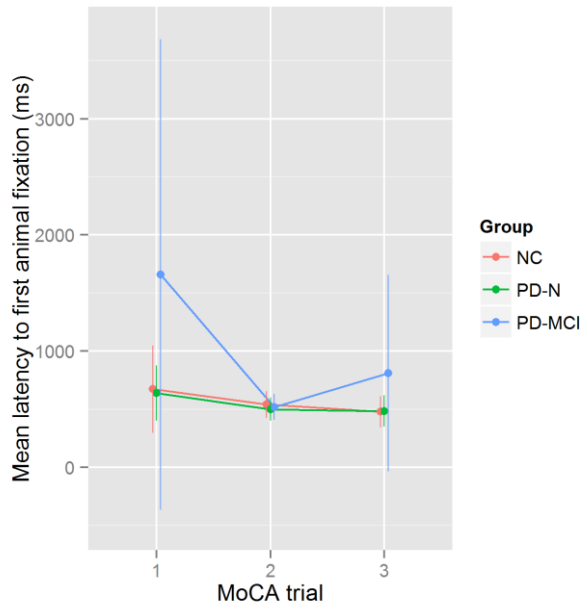


Figure 5-6. Mean latencies to fixation on the first animal, per trial of MoCA, stratified according to group.

5.2.3.8 Latency to the first fixation per animal

There were 405 individual recordings for this parameter. Due to unacceptable quality, 41 recordings were excluded. Fewer recordings were excluded from the NC group (eight recordings) than from either PD group (15 from the PD-N group and 18 from the PD-MCI group).

The mean time before fixating on any animal was not significantly different between the groups: 1 007 ms for NC participants, 852 ms for PD-N participants and 1 135 ms for PD-MCI participants.

5.2.3.9 Latency to vocal response

A total of 405 responses were recorded. Forty-three recordings were excluded, the details of which (stratified by group) are in Table 5-9 below.

Table 5-9. Classification of excluded recordings from the vocal response parameter.

	NC	PD-N	PD-MCI	Total
Technical difficulties	8	15	18	41
Required prompting	1	0	1	2
Total	9	15	19	43

The mean latencies to vocal response were 1 252 ms (\pm 339), 1 092 ms (\pm 298) and 1195 ms (\pm 295) for NC, PD-N and PD-MCI participants, respectively. There was no statistically significant difference between the groups.

5.2.4 Discussion

5.2.4.1 Time to trial completion

The time taken to complete the MoCA task was not significantly different between the three study groups. Because the task was relatively simple, it may not have been sufficiently cognitively-demanding to uncover any difference, if present, between the groups (i.e., a ceiling effect of the current task).

PD patients have been shown to demonstrate deficits in naming tasks generally (Tröster, Stalp, Paolo, Fields, & Koller, 1995) and confrontational naming tasks specifically (Hong et al., 2014). There did not appear to be a significant relationship between oculomotor function/planning and performance on the task in participants in this study. This is in accordance with findings by Bodis-Wollner and Jo who suggested that naming required less executive processing, compared

with other language skills such as comprehension and fluency (2006). It would perhaps have been helpful to have included a PD-D group in this study to allow for a more comprehensive examination of such results.

5.2.4.2 Correctness

Most participants correctly identified the names of the nine animals presented. The most common errors were confusing the rhinoceros for a hippopotamus and vice versa. Stratifying PD participants by cognitive status failed to yield any significant differences between either PD-N or PD-MCI and the NC group. In the only other published study (Del Brutto & Wright, 2015) that specifically looked at animal naming sub-scores of the MoCA, authors found three covariates to significantly positively influence the animal naming sub-score: younger age (60-74 years), sex (men) and higher level of education (more than primary education). However, the study population was from rural Latin American communities (using the Spanish MoCA), and cognitive status was not controlled for *per se*.

When Nasreddine and his colleagues designed the MoCA, it was their intention to use drawings of “low-familiarity” animals in order to assess language (2005). Familiarity, however, is relative to sociocultural context, which ought to be taken into consideration when administering such a task. Even so, it is unlikely to explain the results found in this study or the pattern of most mistakes (since participants equally confused the rhinoceros and hippopotamus, as opposed to exclusively being familiar with one but not the other).

5.2.4.3 Saccade amplitude

NC participants made significantly bigger saccades compared with participants in either PD group. This is in agreement with findings by Matsumoto *et al* (2011).

Hypometric saccades are characteristic eye movement abnormalities in PD patients (Anderson & MacAskill, 2013). PD patients exhibit reduced voluntary saccadic amplitudes (particularly when looking upwards) early in the course of the disease. As the disease progresses, especially when dementia intervenes, hypometric voluntary as well as reflexive saccades become apparent (MacAskill et al., 2012). This pattern of aberrant eye movements in early PD is thought to be related to basal ganglial loss of dopaminergic neurons (Terao et al., 2011).

5.2.4.4 Number of fixations and their duration

There was no difference in the number of fixations or fixation duration in PD-N and PD-MCI participants compared with NC participants. This is partially contrary to what Matsumoto and colleagues from Japan found; PD subjects in their study took significantly longer to fixate on simple figures compared with controls, but this difference was lost when PD subjects fixated on more complex figures (2011).

The observed differences between the two studies might, at least in part, be ascribed to a number of essential methodological differences. The study by Matsumoto *et al* (2011) did not stratify for cognitive status for PD subjects, and subjects were given time-limits and were asked to memorise the figures (i.e., a delayed task). The present study, on the other hand, separated PD-N from PD-MCI participants, was not timed and participants were asked for instantaneous responses (i.e., an immediate task).

Methodological differences do not offer a full explanation, however. The impression of most participants in this study was that the MoCA animal identification task was one of the simplest to perform. Extrapolating findings from

the Japanese subjects to this study, one would then expect fixation durations akin to those when subjects were shown the simple, rather than the complex, figures. That the “low-familiarity” of the MoCA animals added to the task’s difficulty up to a level comparable to memorising complex figures seems therefore unlikely.

5.2.4.5 Areas of fixation interest

To our knowledge, this is the first study to examine eye movements in this task. Although there were no group-specific patterns in the generated area of fixation interest, several observations—upon the performance of the study population as a whole—are of interest. First, participants principally focused their fixations on the animal’s head, the area perceived to be most informative. The notable exception was the giraffe, whose conspicuously-long neck was sufficiently informative for most participants to recognise the drawing as that of a giraffe’s. In fact, there were almost no fixations below the giraffe’s torso/lower neck (see Figure 5-7).

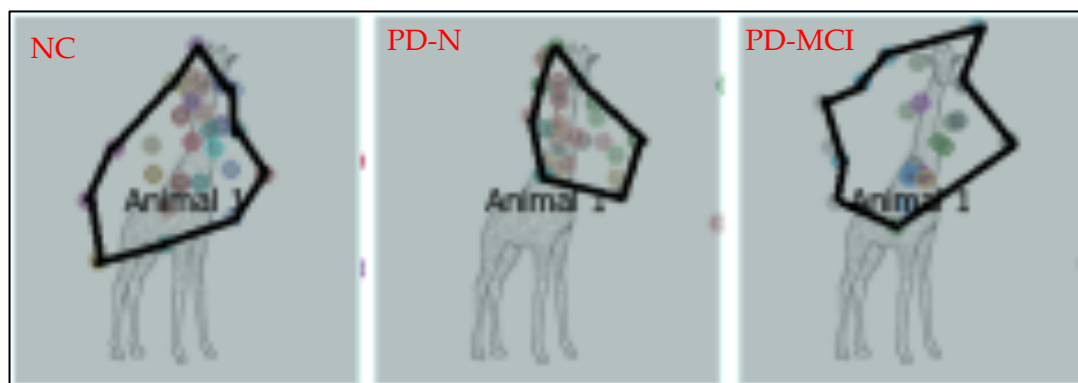


Figure 5-7. Areas of fixation interest, in each group, pertaining to the giraffe of MoCA trial 2.

Extrapolating to other animals with ‘distinctive’ features, another pattern emerged (see Figure 5-8). Animals with ‘distinctive’ features (e.g., the long neck of the giraffe or the long tail and bipedalism of the kangaroo) had relatively smaller associated areas of fixation interest. In contrast, animals with less distinctive

features (e.g., the donkey or pig) had comparatively larger associated areas of fixation interest. Participants presumably required more careful oculomotor exploring of these animals' bodies in order to determine at which animal they were looking (e.g., donkey vs. horse or cow).

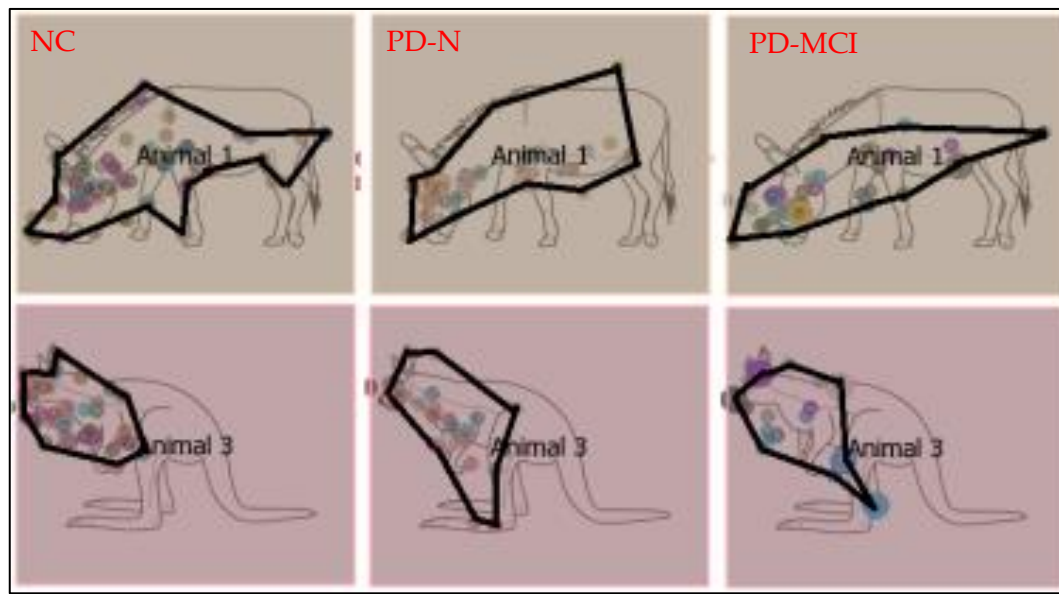


Figure 5-8. Areas of fixation interest for a 'less distinctive' animal (upper panel) vs. 'more distinctive' animal (lower panel) in each group.

From previous studies, it seems that the familiarity of the animal determines whether participants correctly identify it, taking into account the sociocultural influence on performance. For example, a common mistake made by elderly subjects in rural Ecuador (Del Brutto & Wright, 2015) was mistaking the rhinoceros on the Spanish MoCA (equivalent to MoCA trial 1 in this study) for a cow (i.e., a much more familiar quadrupedal animal to them).

The animal's morphology (regardless of familiarity), on the other hand, appears to dictate the pattern of fixations (i.e., duration, location and number) when trying to correctly identify the animal drawing. The antithesis of this (i.e., restricting the time allowed to visually explore two morphologically similar animals) should result in decreased accuracy of animal identification. This latter notion has been

examined in a recent study by Wu and colleagues (2015). In their study, healthy subjects sat in front a screen where two stimuli, arranged side-by-side, were presented simultaneously for a period of only 400 ms. Participants were asked to always look towards the dog. They performed better when the two animals presented were morphologically different (e.g., dog vs. bird) than when morphologically similar (e.g., dog vs. cat).

Although participants did not necessarily fixate on the unique features of 'more distinctive' animals, peripheral vision might have contributed to recognising the animal, and fixations on the head were perhaps made to confirm that. There is compelling evidence in the literature that humans are particularly efficient at recognising certain categories of visualised scenes. This observation has been confirmed in both behavioural (Thorpe, Fize, & Marlot, 1996) and electrophysiological (Rousselet, Mace, Thorpe, & Fabre-Thorpe, 2007) studies. Part of the underlying neurophysiology of this phenomenon has been attributed to faster eye movements, especially towards "biological stimuli", such as human faces (Crouzet, Kirchner, & Thorpe, 2010) and animals (New, Cosmides, & Tooby, 2007), as well as a remarkable accuracy of detecting the same object categories using extrafoveal (i.e., peripheral) vision (Thorpe, Gegenfurtner, Fabre-Thorpe, & Bulthoff, 2001).

Caution is warranted in extrapolating the above observations to those of the present study. This is because most of these previous experiments utilised coloured photographs of animals in natural scenes whilst monochrome animal drawings were used herein. Also, most of these earlier studies did not require substantial semantic processing (i.e., participants were asked to look towards the animal as opposed to naming it). Furthermore, suggestions made earlier are in contrast to findings by Kietzmann and his colleagues (2011). They found

participants to have made eye movements (i.e., visual attention) prior to recognising what the ambiguous image represented (i.e., object awareness).

It is important to recognise the difference in the methodology between the present study and that of Kietzmann and colleagues (2011). Whereas I presented clear drawings of animals and asked participants to name them, they showed participants ambiguous stimuli and asked participants if they recognised the object and later to name it (Kietzmann et al., 2011). To account for this apparent discrepancy, I suggest a combined sequence of events that explains findings of both studies: fixations precede object recognition; once identified, this is followed by confirmatory eye movements to collect further evidence before an answer is spoken.

There is also an alternative hypothesis to explain the smaller areas of fixation interest for specific animals; the order in which the three animals were seen in the MoCA trial appears to affect their associated area of fixation interest. For animals seen last, participants may have utilised the luxury of time and peripheral vision whilst fixating on the first two animals. Therefore, participants may not have needed to fixate on them as much. While perhaps plausible, this hypothesis does not explain why the lion (the first animal seen by most participants in MoCA trial 1; figure not shown) and the giraffe (the first animal seen by most participants in MoCA trial 2) had smaller areas of fixation interest compared to the subsequent animals in their respective trials.

5.3 JLO task

5.3.1 Background

Available literature on the performance on the JLO test by PD patients is largely descriptive, and this has been described elsewhere in this thesis (see Chapter 4). How PD patients reach their final answers (whether correct or erroneous), for instance by analysing available data to unveil any underlying search strategies, has seldom been explored (Montse, Pere, Carme, Francesc, & Eduardo, 2001).

Employing eye tracking methods while PD participants attempt several JLO items may prove valuable in further characterising error-patterns—potentially shedding light on a ‘faulty’ strategy stemming from a higher executive dysfunction common in PD.

5.3.2 Methods

5.3.2.1 Definitions

The JLO task was made up of 15 trials (five Practice and ten Test trials). The participant was asked to correctly identify which of the 11 reference lines matched the pair of test lines (‘stimuli’). The first stimulus (S_1) refers to the left-most line of the pair. For analysis, a rectangular region was specified around the stimuli, and was referred to as the ‘Working Area’. Another rectangular region was specified around the reference lines, and was named the ‘Scanning Area’ (see Figure 5-9 for an illustration).

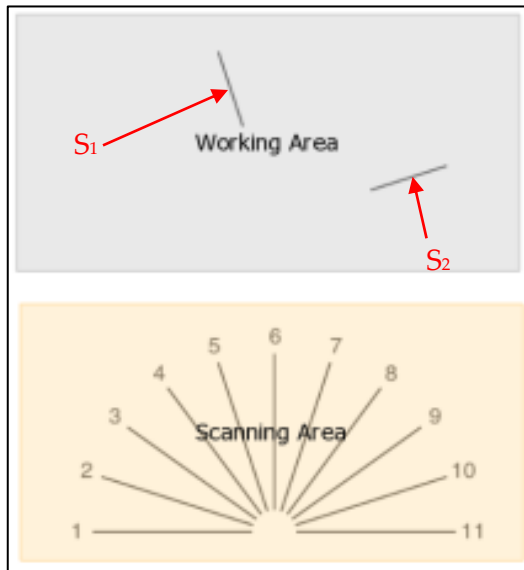


Figure 5-9. Defining JLO task-related parameters.
 S_1 = the first stimulus; S_2 = the second stimulus.

5.3.2.2 Protocol

The instructions to the task were explained verbally before the start of the task. All trials were chosen from the H form of the JLO, and were presented in the same order. Participants were not specifically instructed to start with a specific (S_1 vs. S_2) stimulus.

Immediately prior to the presentation of each trial, participants were asked to fixate on six pre-selected calibration-check targets which were presented one-at-a-time. The targets were distributed to give adequate coverage over an area marginally larger than the field in which the trials were presented (19° horizontally by 19° vertically). The targets were presented quasi-randomly; the last calibration target was always the (0° , 3°) target (i.e., the neutral point), while the first five appeared in a randomised order. See Figure 5-10 for an example.

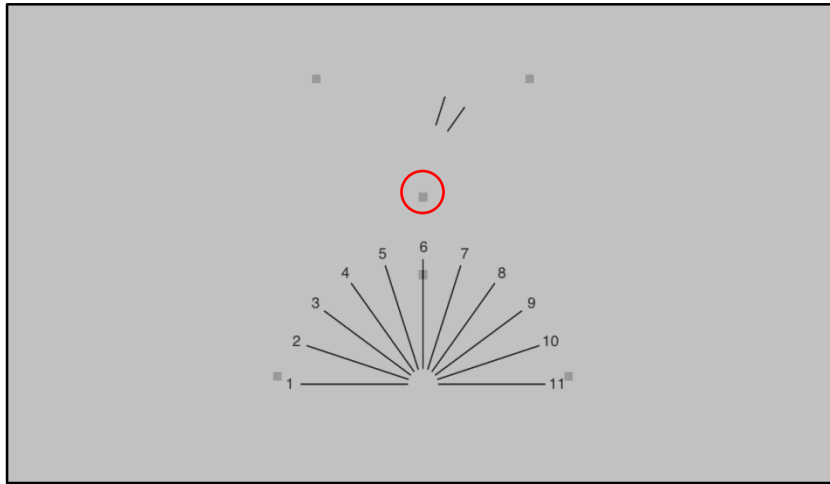


Figure 5-10. An example JLO trial overlaid with calibration-check target locations. The circled target was always the last to be presented (i.e., the neutral point).

Participants were initially shown five consecutive Practice trials, and were asked for responses. The stimuli in the Practice trials were a pair of full-length lines. Correct responses were affirmed, while incorrect responses were corrected, and participants were encouraged to look at the practice trial again to try to identify why they originally chose the wrong responses. Once the participant was satisfied with the answer, the next Practice trial was presented. The start of each Practice trial was researcher-initiated via a keyboard. Eye movements were not recorded during Practice trials.

Once the Practice trials were completed, a wireless keyboard was activated, and participants were asked to initiate the Test trials by pressing any key. As automated via PsychoPy, this lead to the presentation of each Test trial, as well as a simultaneous 400 ms A-note beep (which was used as a reference when recorded voice responses were played on Audacity® for analysis). The trial was ended by the researcher once answers for both stimuli were provided. The ten Task trials were chosen as a selected representative subset of trials, in an ascending order of difficulty (namely, H-1, H-3, H-12, H-13, H-17, H-20, H-24, H-27, H-28 and H-29).

The stimuli in the Test trials represent a pair of partial-length lines (only a third of the line was shown). The protocol is illustrated diagrammatically in Figure 5-11.

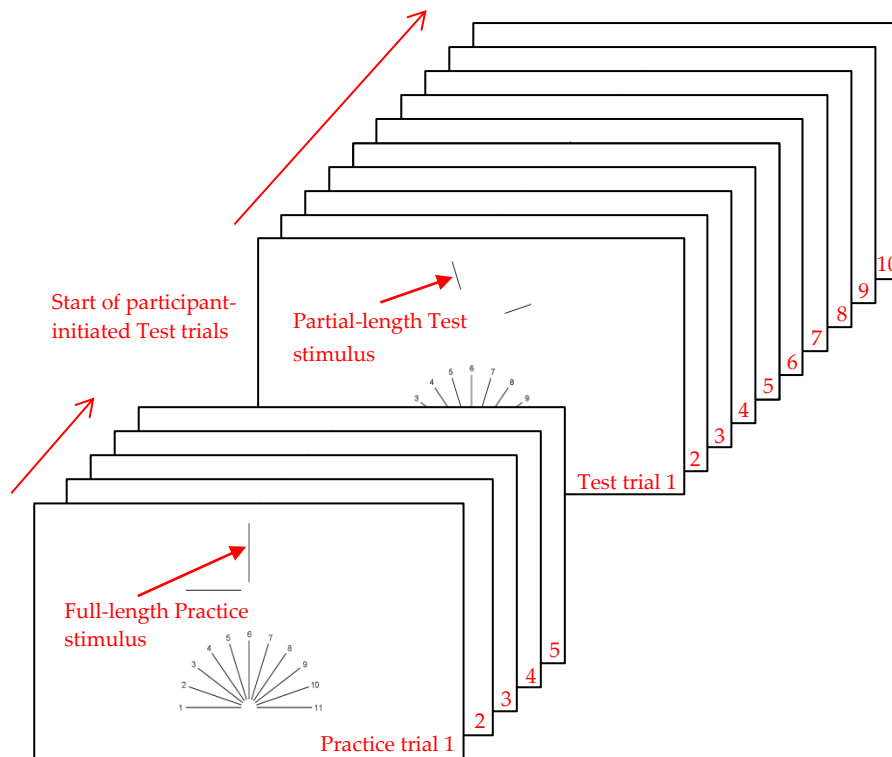


Figure 5-11. JLO task protocol.

5.3.2.3 Correctness

Participant responses were collected twice; notes were taken during task performance, and recorded voice responses were evaluated once all data were collected. Participants were permitted to self-correct, but no hints were provided. Participants scored two points for correctly identifying both stimuli, one point for correctly identifying one stimulus but not the other, and zero points for two incorrect responses.

5.3.2.4 Error analysis

Incorrect responses were analysed using two systems: line location and the Ska classification (see Table 1 in Chapter 4). Because stimuli in Test trials were presented as partial-length lines, this allowed for each partial-length line location to be classified as high, middle or low.

5.3.2.5 Performance on Practice trials

Participant performance on Practice trials was tested for any correlation with performance on Test trials. Akin to assessing Test trials, each Practice trial was assigned two points for correctly identifying both stimuli without prompting from the researcher, one point for correctly identifying one stimulus but not the other, and zero points for two incorrect responses.

5.3.2.6 Saccade and fixation data

An upper limit of 26.9° (maximum possible amplitude) was applied to saccade amplitude. This provided complete coverage of the area in which the JLO task was presented. The number of fixations, rather than saccades, was chosen to be reported here as it was thought to be more reflective of the cognitive process of trying to correctly identify both stimuli. Each fixation made by each participant contributed to the heat map (as shown in a coloured timescale) relative to its duration. Heat maps were generated automatically by BeGaze™. Coloured timescales (in ms) were manually set to a fixed maximum for all trials in order to allow for meaningful comparisons whilst ensuring optimal presentation of the trials.

5.3.3 Results

5.3.3.1 Overview

Acceptable data were available for a total of 43 participants who completed the JLO task: 15 NC, 15 PD-N and 13 PD-MCI. Data for one PD-MCI participant were only recorded for nine Test trials. Several parameters (see below) were recorded, each of which had certain quality-assurance criteria. Participants' data falling short of these criteria were discarded. Therefore, depending upon the parameter's criteria, the number of participants may not always be equal to the total number of participants completing the task.

5.3.3.2 Correctness

Only a few participants were able to correctly identify both stimuli in each of the ten Test trials. The average correct response rates were still high, nonetheless: 93% (± 6) for NC, 88% (± 12) for PD-N and 87% (± 11) for PD-MCI. The difference between the groups did not reach statistical significance. Participants made more errors in later trials (especially H-27) compared with earlier trials (see Figure 5-12). All participants correctly identified both stimuli of the H-3 trial.

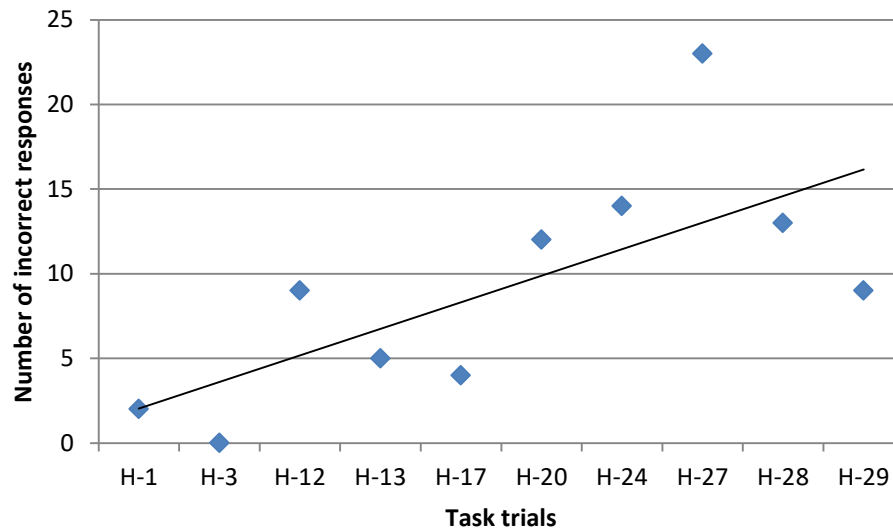


Figure 5-12. The total number of incorrect responses per JLO trial across all participants. Given trials were presented in an ascending order of difficulty, it was expected to detect more incorrect responses in later trials ($R^2 = 0.49$, $p = 0.02$).

5.3.3.3 Error type

The overall most common line location associated with erroneous identification was low partial-lines. The per-group differences are shown in Table 5-10. Using the Ska classification, error types QO_1 and QO_3 were by far and away the most common. PD participants also made a few other error types (see Table 5-10).

Table 5-10. Details of error types made by participants in the JLO task.

	Line location			Ska classification					Error total*
	L	M	H	QO_1	QO_3	QO_4	V	IQO	
NC	8	7	6	9	12	0	0	0	21
PD-N	18	7	11	20	9	6	1	0	36
PD-MCI	13	11	10	20	9	4	0	1	34
Total	39	25	27	49	24	5	1	1	

* = Total number of errors per group; L = low; M = middle; H = high.

5.3.3.4 Performance on Practice trials

Most participants (86%) correctly identified eight or more out of the ten Practice stimuli. A Pearson correlation failed to reveal a significant relationship between performance on Practice and Test trials.

5.3.3.5 Saccade amplitude

Mean saccade amplitudes for all participants are presented in Table 5-11. NC participants made marginally bigger, albeit not statistically significant, saccades compared with PD participants.

Table 5-11. Saccade amplitudes across the study groups performing the JLO task.

	Mean (°)	SD	ANOVA	<i>p</i>
NC	5.9	0.9	$F_{2,40} = 0.45$	0.64
PD-N	5.7	1.1		
PD-MCI	5.5	1.0		
Total	5.7	1.0		

5.3.3.6 Heat maps

As a whole, participant fixation patterns were similar across all JLO trials. In the Scanning Area, most fixations were made on the distal ends of the reference lines. Fixations in the Working Area, on the other hand, appeared to vary between trials. These patterns were observed whether the response was correct or incorrect. Below are the heat maps of three representative JLO trials: H-3 trial (both stimuli identified correctly by all participants), H-27 (trial in which most errors occurred), and H-24 (which had a few erroneous responses that were distributed equally between NC, PD-N and PD-MCI participants).

Figure 5-13 shows the H-3 trial, both stimuli of which were correctly identified by all participants. Across all groups, fixations in the Scanning Area are most concentrated on the distal ends of the reference lines, whereas fixations in the Working Area were more concentrated on the proximal end of S_2 (and the distal end of S_1).

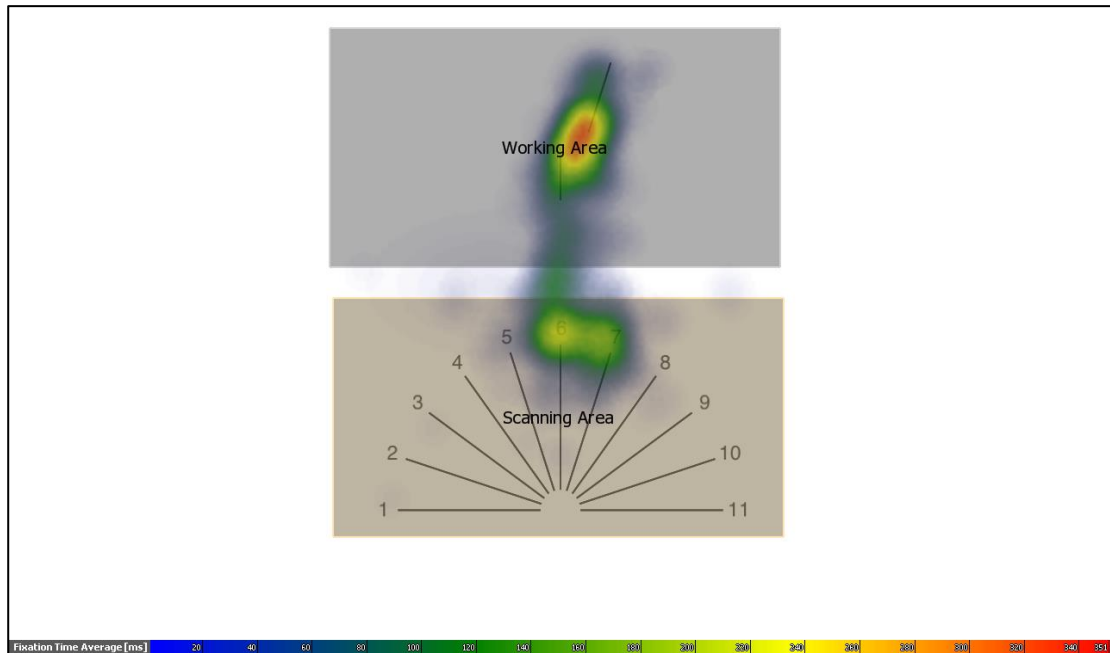


Figure 5-13. A heat map of combined fixations of trial H-3. The correct responses were lines 6 and 7.

Figure 5-14(a) and Figure 5-14(b) show fixation densities of the Correct and Incorrect subjects, respectively, for the H-27 trial. The pattern of fixations in the Scanning Area is similar; participants fixated most on the distal ends of the reference lines (albeit the wrong ones in the case of the Incorrect group). Fixations in the Working Area were longer (i.e., from the provided time-scale) and more diffuse across both stimuli, compared to fixations in the Scanning Area.

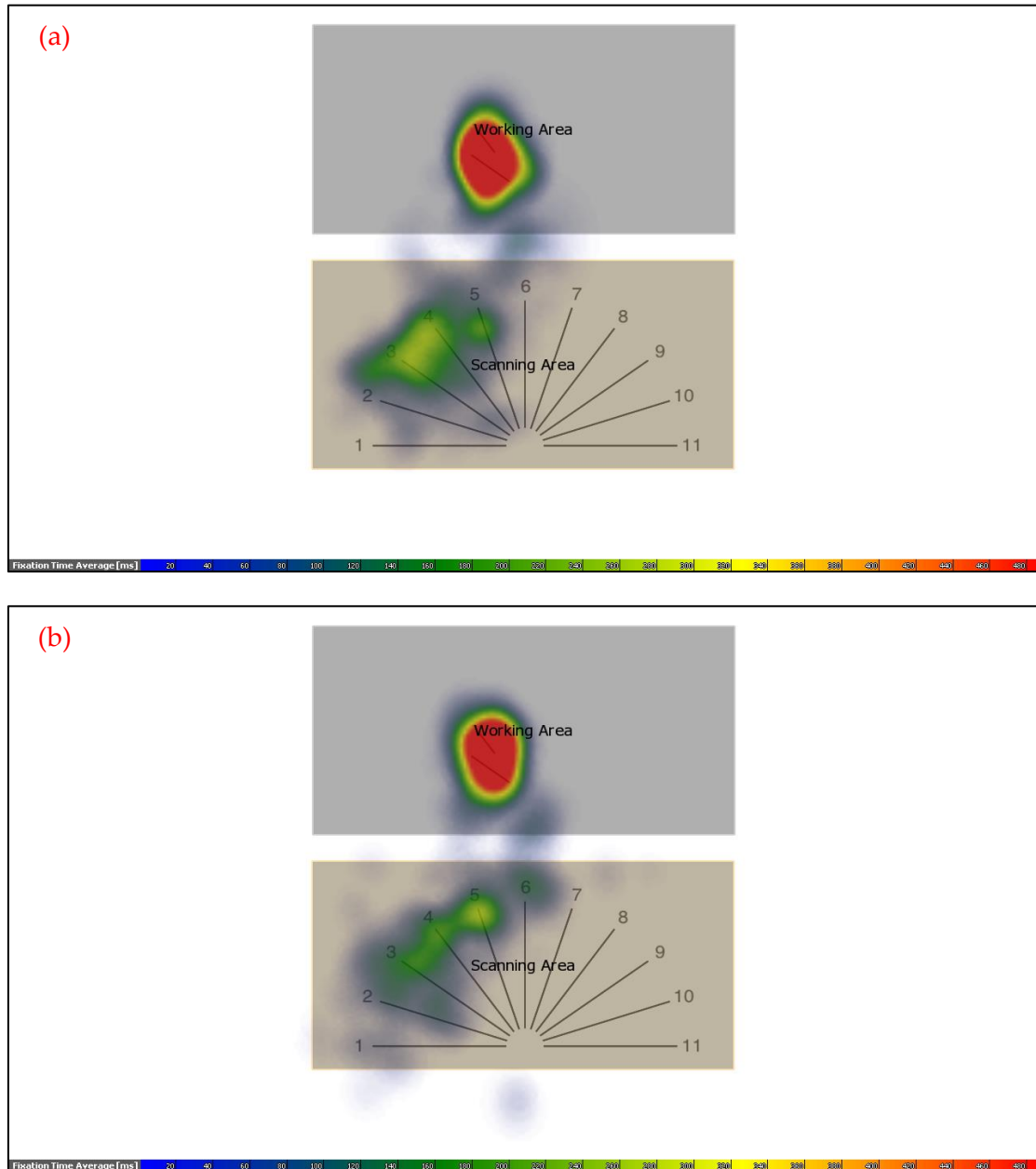


Figure 5-14. Trial H-27 as performed by the Correct subjects (a) and Incorrect subjects (b) across all three groups. The correct responses were lines 3 and 4.

Finally, heat maps for the Correct and Incorrect subjects of trial H-24 are shown in Figure 5-15(a) and Figure 5-15(b), respectively. Erroneous responses from the H-24 trial, for which acceptable eye movement data were available, were distributed equally between the study groups (three responses for each of NC, PD-N and PD-MCI groups). Similar to trial H-27, participants fixated most on the distal ends of reference lines (albeit the wrong ones in the case of the Incorrect group). Fixations

in the Working Area were longer (i.e., from the provided time-scale) and more diffuse across both stimuli, compared to fixations in the Scanning Area.

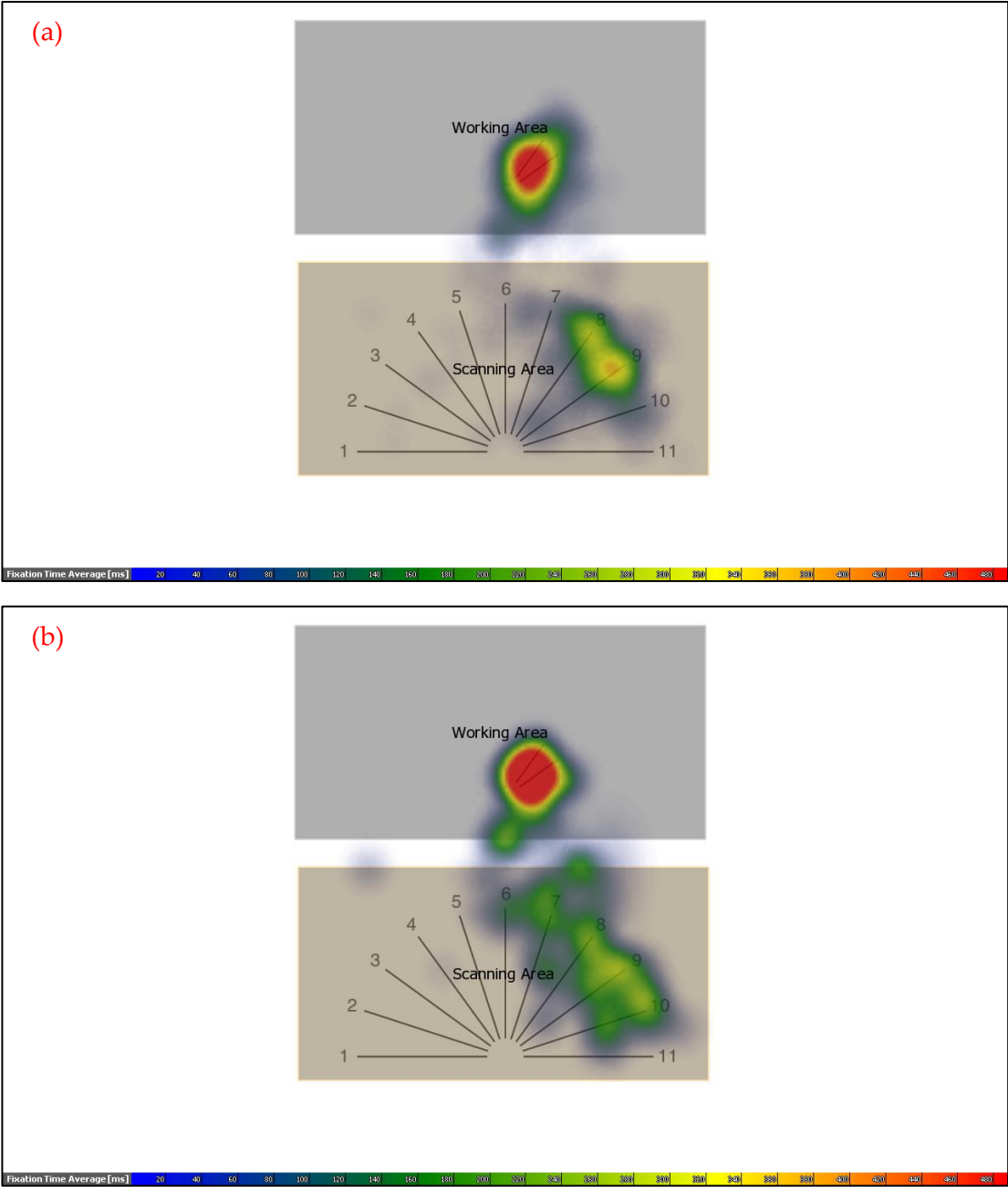


Figure 5-15. Trial H-24 as performed by the Correct subjects (a) and Incorrect subjects (b) across all three groups. The correct responses were lines 8 and 9.

5.3.4 Discussion

5.3.4.1 Overview

The proportion of trials for which acceptable data are available was slightly less than that of the MoCA task. This is to be expected, however, as the JLO task was harder and involved a higher number of trials. Also, only selected items of the actual JLO neuropsychological test were presented in this study. This, in turn, limits the generalisability of the results.

5.3.4.2 Correctness

As expected, participants made more errors in the later trials, most likely as a result of increasing trial difficulty. Although performance slightly worsened going from NC, to PD-N and finally to PD-MCI participants, the difference did not reach statistical significance ($p = 0.21$).

Findings here differ from those reported in the literature whereby PD patients score significantly lower in the full JLO task, compared to matched controls (Hovestadt, de Jong, & Meerwaldt, 1987; Levin et al., 1991; Montse et al., 2001). One possible explanation of this discrepancy is that for practical reasons, the JLO task in this study only included a few selected trials (10 items), instead of the entire task (30 items). This, in turn, could have mitigated an otherwise significant difference between the groups had more trials been included.

Owing to the large number of tasks participants were expected to complete in this study, only selected items of several neuropsychological tests were utilised. This was done with the intention of avoiding participant fatigue and to conserve testing time. The JLO alone takes an estimated 21 minutes to be administered in

full (Lundin & DeFilippis, 1999), without the additional time required for eye movement recording procedures. Recently, Gullett and his colleagues demonstrated the reliability of the JLO short forms, OF and EF (15 items each) and TF (20 items), in patients with PD (2013).

5.3.4.3 Error types

Two classification systems were employed to categorise erroneous responses in the JLO task. The line location classification revealed low partial-lines to be associated with many more incorrect responses, compared with middle and high partial-lines.

Using the Ska classification (Ska, Poissant, & Joannette, 1990), several patterns emerged. The most common error types were QO₁ and QO₃. QO₁ errors were predominantly made by PD participants. QO₃ errors were slightly more common in NC participants than either PD group. Other error types (several QO₄, one V and one IQO errors) were exclusive to PD participants. These findings are generally in keeping with the published literature (Finton, Lucas, Graff-Radford, & Uitti, 1998; Montse et al., 2001). The finding by Finton *et al* that H type errors were also common in PD patients (1998) cannot be supported or refuted by findings of the present study. This is because my study did not include any horizontal Test stimuli (i.e., parallel to reference lines 1 or 11), and therefore, any such potential deficits in PD participants could not be ascertained.

5.3.4.4 Heat maps

Close inspection of the fixation heat maps generated for all JLO trials reveals some interesting and persistent patterns. Most fixations in the Scanning Area are made to the distal ends of the reference lines (i.e., close to the numbers). This is contrary

to my hypothesis, whereby most Scanning Area fixations were expected to occur more proximally on the reference lines to enable spatial comparison and narrow down the choices to three or four possibilities. These results bear similarities to findings by Mosimann and colleagues who examined clock reading in a group of AD patients (2004). Whereas healthy controls focussed on the distal end of each clock hand (i.e., closest to the number), AD patients' fixations were less organised.

The observed pattern was consistent across the study groups and also across all JLO trials (regardless of the position of the Test stimuli: low, middle or high). One could contend that participants in fact made more fixations on the choice numbers adjacent to the distal ends of the reference lines prior to vocalising their responses. However, this is unlikely as participants appeared to make a small number of fixations to the number once a decision on the answer(s) had been reached. This observation is particularly evident in tasks where the choice numbers are not as close as they are in the JLO task (see the MR task next). Perhaps a more plausible explanation is that participants subconsciously try to extrapolate a line that connects the reference line to the very stimulus in question. This is especially apparent in reference line 6 and S₁ (see Figure 5-16 below) of task H-3 (in which all participants' responses were correct). A line of fixations can almost be seen connecting the distal end of reference line 6 and the proximal part of S₁.

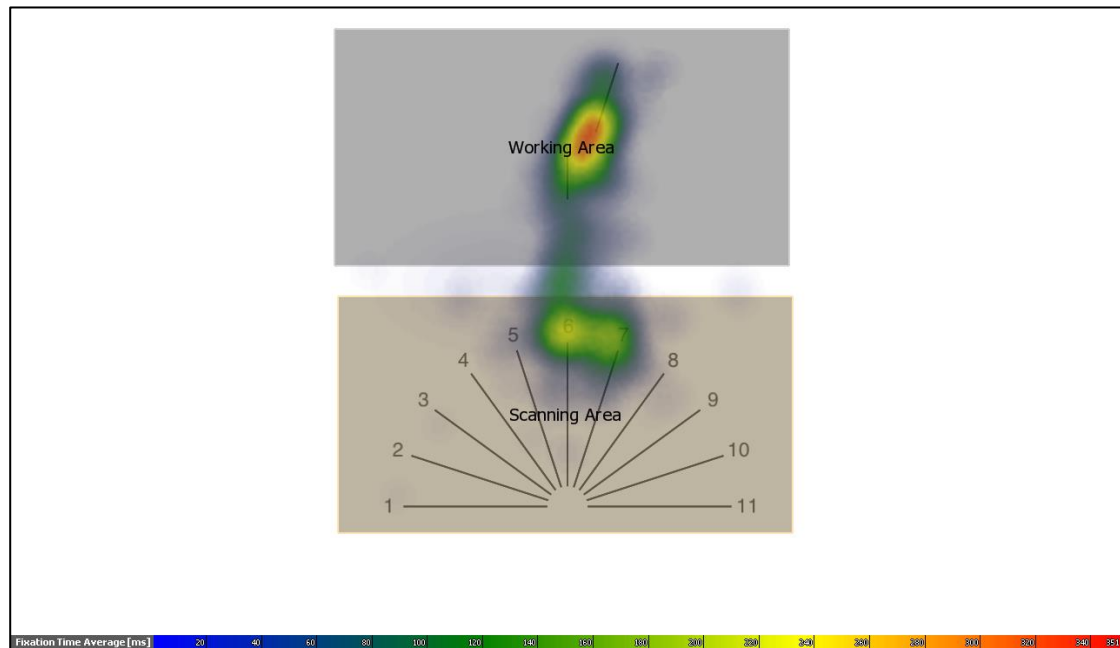


Figure 5-16. JLO trial H-3: a faded green line (of fixations) can almost be seen connecting the distal end of reference line 6 and the proximal part of S1.

The main difference in the heat maps of the Correct and Incorrect groups was the fixation density (i.e., a product of both the number of fixations and the duration of each fixation) of their respective maps; the Incorrect group had increased density heat maps compared with the Correct group (see Figure 5-14 and Figure 5-15).

5.4 MR task

5.4.1 Background

The most relevant data to date on the performance in MR by PD patients come from a single case-control study from Christchurch, New Zealand. In this study, PD patients and matched controls underwent a battery of neuropsychological tests among which was the MR. PD patients scored significantly lower on the MR, compared with control subjects (McKinlay, Grace, Dalrymple-Alford, & Roger, 2010). Another more recent, although not directly related, study found MR scores

to differentiate PD patients with freezing of gait (lower MR scores) from those who do not freeze (Nantel, McDonald, Tan, & Bronte-Stewart, 2012). However, a matched control group was lacking in the study. Eye tracking during the MR task was included to provide insight into possible strategies utilised by PD participants (PD-N vs. PD-MCI) compared with healthy controls.

5.4.2 Methods

5.4.2.1 Definitions

The MR task was made up of 11 trials. Participants were required to complete the visual pattern presented in the 2x2 matrix by selecting the correct option from a multiple choice array. Correctly choosing the missing pattern was required to score a point and qualify the trial as 'correct'. For analysis, a rectangular region was specified around the 2x2 matrix, and was named the 'Scanning Area'. Another rectangular background was specified around the multiple choice panel, and was called the 'Working Area'. See Figure 5-17 for an illustration.



Figure 5-17. Defining MR task-related parameters.

5.4.2.2 Protocol

The task instructions were given verbally before the start of the task. Brief written instructions (“Say the number of the correct choice”) were also projected onto the screen prior to the start of each trial. Eleven selected trials were presented in an increasing level of difficulty (namely, MR-0A, MR-04, MR-05, MR-10, MR-13, MR-16, MR-19, MR-20, MR-21, MR-23 and MR-24). All trials were presented in the same order across subjects.

Immediately prior to the presentation of each trial, participants were asked to fixate on six pre-selected calibration targets which were presented one-at-a-time. The targets were distributed to give adequate coverage over an area marginally larger than the field in which the trials were presented (20° horizontally by 15° vertically). The targets were presented quasi-randomly; the last target was always the (0°, 9°) target (i.e., the neutral point), while the first five appeared at no particular order. See Figure 5-18 for an example.

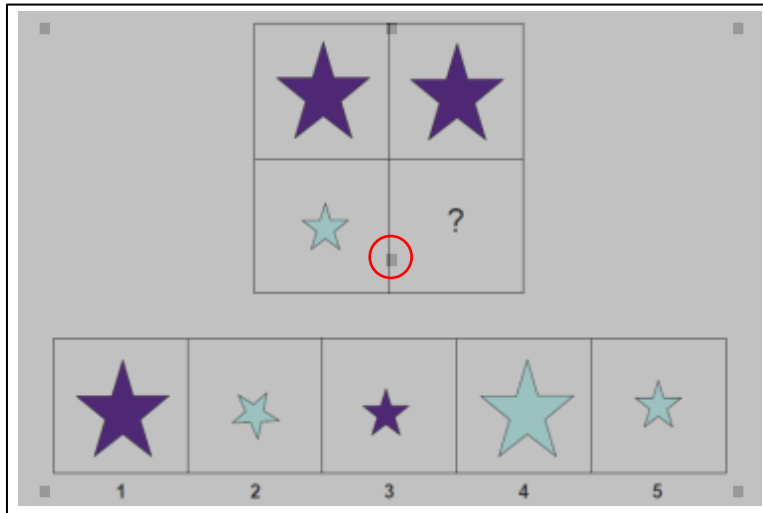


Figure 5-18. An example MR trial with overlaid calibration-check targets. The circled target was always the last to be presented (i.e., the neutral point).

All trials were researcher-initiated by pressing any key on the keyboard. This led to the presentation of the trial, as well as a simultaneous 400 ms A-note beep (which was used as a reference when recorded voice responses were played on Audacity® for analysis). The trial was ended by the researcher once an answer was provided. The protocol is illustrated diagrammatically in Figure 5-19.

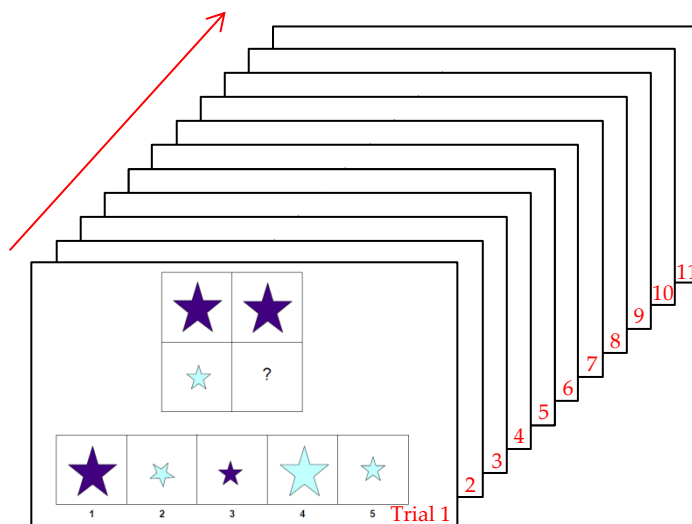


Figure 5-19. MR task protocol.

5.4.2.3 Correctness

Participant responses were evaluated twice; notes were taken during task performance, and definitive recorded voice responses were evaluated once all data were collected. Participants were allowed to self-correct, but no hints were provided. Participants scored one point for correct responses and zero points for incorrect responses.

5.4.2.4 Saccade and fixation data

An upper limit of 25° (maximum possible amplitude) was applied to saccade amplitude. This provided complete coverage of the area in which the MR task was presented. The number of fixations, rather than saccades, was chosen to be reported here as it was thought to be more reflective of the cognitive process of trying to correctly identify the missing pattern. Similar methods to the JLO task were used to generate heat maps.

5.4.3 Results

5.4.3.1 Overview

A total of 45 participants completed the MR task: 16 NC, 15 PD-N and 14 PD-MCI. Physical fatigue was the most common reason for not completing the MR task. Several parameters (see below) were recorded, each of which had certain quality-assurance criteria. Participants' data falling short of these criteria were discarded. Therefore, depending upon the parameter's criteria, the number of participants may not always be equal to the total number of participants completing the task.

5.4.3.2 Correctness

Only a few participants were able to correctly identify the missing pattern in each of the trials. The average correct response rates were still high, nonetheless: 77% (± 14) for NC, 78% (± 19) for PD-N and 67% (± 17) for PD-MCI. The difference between the groups did not reach statistical significance. Participants made more errors in latter trials (especially MR-23 and MR-24) compared with earlier trials (see Figure 5-20). All participants correctly identified the missing pattern in the MR-0A trial.

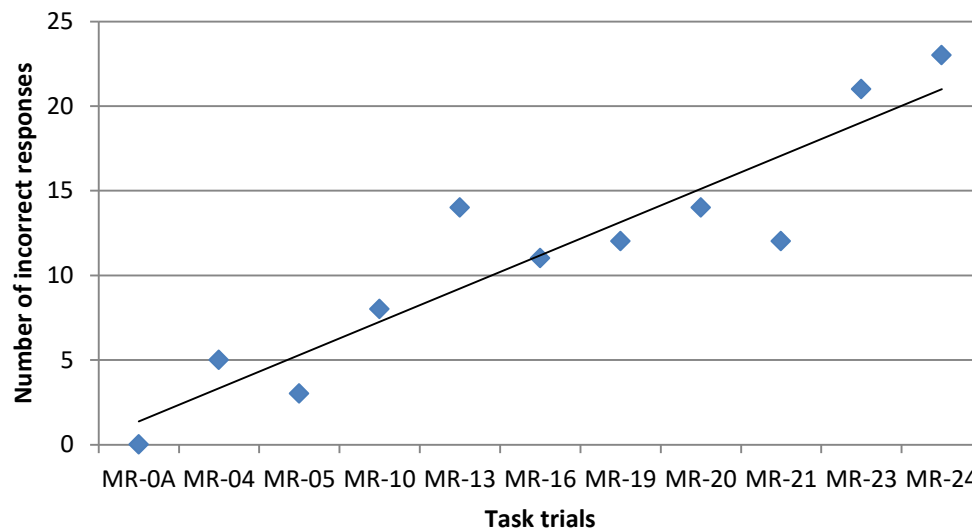


Figure 5-20. The total number of incorrect responses per MR trial across participants. As with the JLO task, trials of the MR task were presented in an ascending order of difficulty, which likely underlies the regression line found based on the number of incorrect responses per trial ($R^2 = 0.86$, $p < 0.001$).

5.4.3.3 Saccade amplitude

Mean saccade amplitude did not significantly differ between study groups: 5.7° (± 0.5) for NC participants, 5.4° (± 0.6) for PD-N participants and 5.4° (± 0.8) for PD-MCI participants.

5.4.3.4 Heat maps

Akin to JLO, general trends of fixation density, across most MR trials, can be visualised. Generally, more time was spent fixating on the Scanning Area than on the Working Area. Within the Scanning Area, fixations on the visual pattern next to the missing one (on the horizontal axis) were longer than fixations on either pattern above or below it. These trends persisted regardless of the response given.

Below are the heat maps of three representative MR trials: MR-0A trial (where the missing pattern was identified correctly by all participants), MR-24 (the trial in which most errors occurred), and MR-21 (which had a few erroneous responses that were distributed equally between NC, PD-N and PD-MCI participants).

Figure 5-21 shows the MR-0A trial, the missing pattern of which was correctly identified by all participants. As stated above, most fixations were in the Scanning Area.

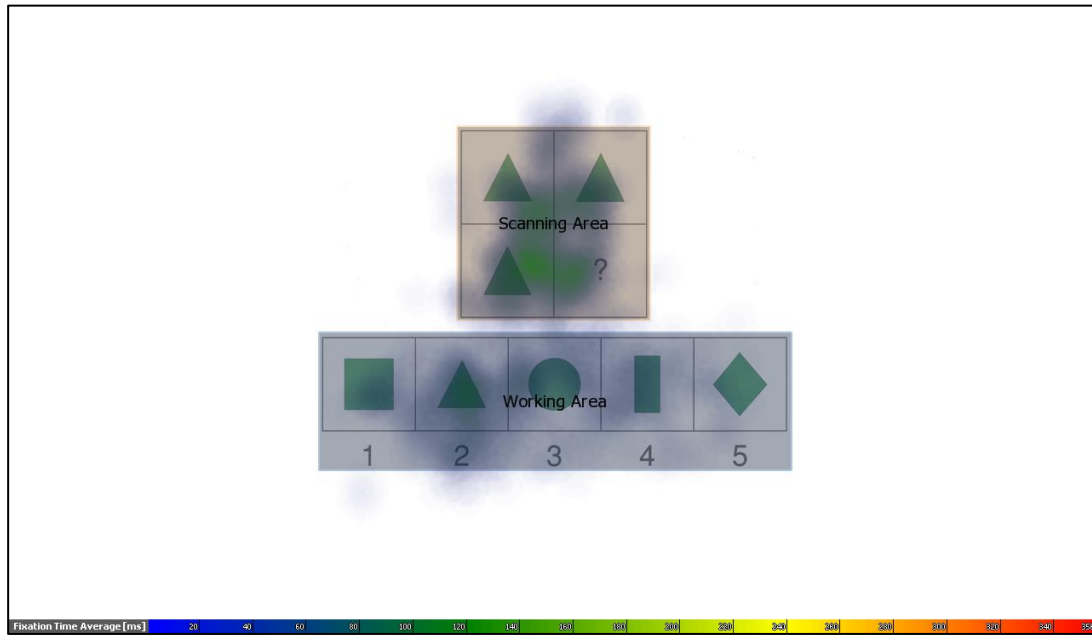


Figure 5-21. A heat map of trial MR-0A (all participant responses were correct). Owing to the short duration in which all participants identified the correct response, this heat map appears 'less dense' relative to the following heat maps (the colour time-scale has been standardised across all heat maps).

Figure 5-22(a) and Figure 5-22(b) show fixation densities of the Correct and Incorrect subjects, respectively, for trial MR-24. Performance on this trial appears to conform to the general trends seen elsewhere in this task. Of note, fixations in the Working Area appear considerably more focussed (on the correct choice) in the Correct subjects compared with the Incorrect subjects. Moreover, almost no fixations occur in the blank quadrant (marked '?') of the Scanning Area.

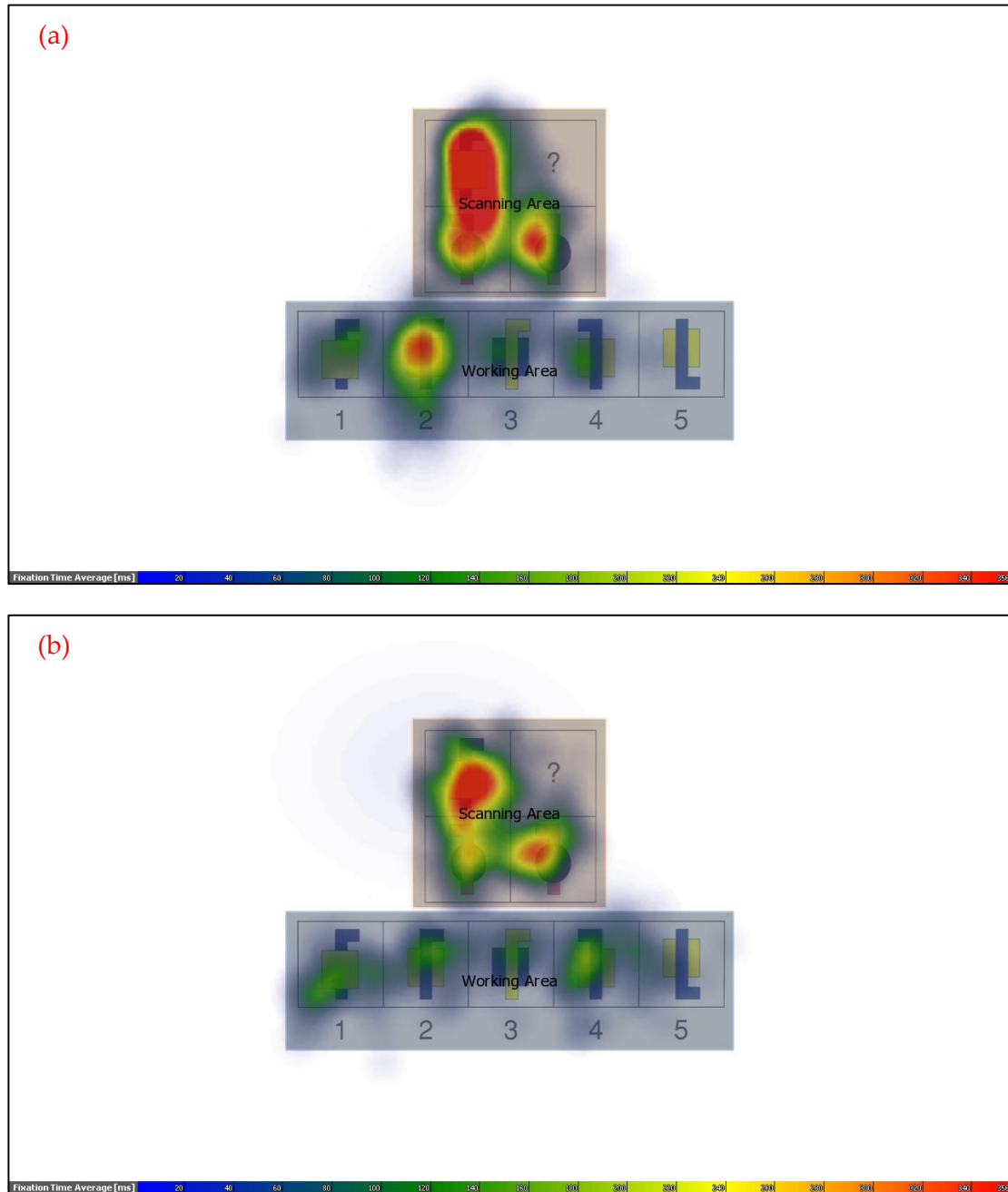


Figure 5-22. Heat maps of the performance of the Correct (a) and Incorrect (b) subjects in trial MR-24.

Lastly, heat maps of trial MR-21 for the Correct and Incorrect subjects are shown in Figure 5-23(a) and Figure 5-23(b), respectively. Erroneous responses from the MR-21 trial, for which acceptable eye movement data were available, were distributed equally between the groups (four responses for each of NC, PD-N and PD-MCI groups). Almost exactly the same fixation patterns, observed in MR-24, are also seen here: more fixations were made in the Scanning Area, especially on

the visual pattern right of the missing one and fixations by the Incorrect subjects were scattered between the possible answers.

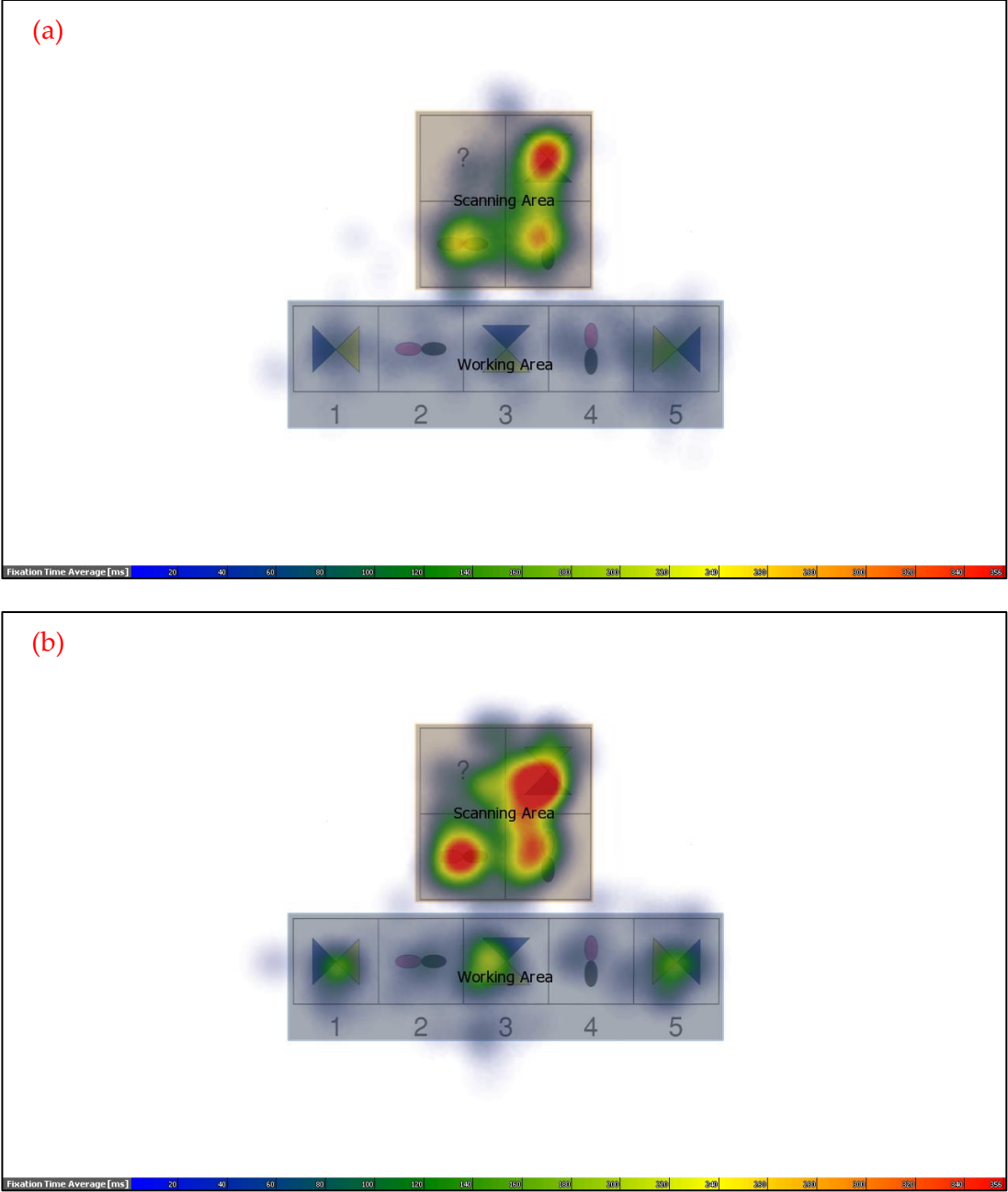


Figure 5-23. Trial MR-21 heat maps: Correct group (a) and Incorrect group (b).

5.4.4 Discussion

5.4.4.1 Correctness

Differences in the proportions of correctly identified patterns among the three study groups did not reach statistical significance. This could be due to a similar scenario with the JLO task: the number of MR items used in this study was insufficient to reveal any differences between the groups. Supporting this explanation are the significantly lower scores of PD patients who performed the full MR (McKinlay et al., 2010) and other similar tests, such as Raven's Coloured Progressive Matrices (Bašić et al., 2004).

Furthermore, more erroneous responses were made in the latter trials, compared with the earlier trials. In fact, the number of errors in the last three trials (i.e., the most difficult trials: MR-21, MR-23 and MR-24) is almost equal to that of all earlier trials combined.

5.4.4.2 Heat maps

Certain global patterns of fixations were evident throughout the MR task. These patterns were often preserved regardless of study group (NC vs. PD-N vs. PD-MCI) and response given (correct vs. incorrect).

The proportion of time spent fixating on the Scanning Area was longer than that on the Working Area. Within the Scanning Area, participants fixated longer on the visual pattern next to the missing one compared with the two visual patterns above or below it.

Fixation behaviour on the Working Area differed according to the response given. The Correct group fixated on the correct choice the longest, and not much else. Fixation durations of the Incorrect group, on the other hand, were divided almost equally between two or three choices. Neither long nor more fixations on the choice number were required before voicing a response. This observation serves as further confirmation that the higher density on the distal ends of references lines of JLO's heat maps represents fixations on the lines and not the choice numbers.

5.5 *Where's Wally?*TM search task

5.5.1 Background

There is a paucity of published literature on the performance of PD patients on search tasks in general. It is known that dynamics (i.e., duration, magnitude, rate and velocity) of human saccades and microsaccades are influenced by the cognitive and attentional demands of a visual task (Otero-Millan, Troncoso, Macknik, Serrano-Pedraza, & Martinez-Conde, 2008). Therefore, the aim of this task was to study the pattern(s) of eye movements in a challenging visual search task in PD participants with normal and impairment cognition, and compare this to the performance of healthy controls.

5.5.2 Methods

5.5.2.1 Overview

All participants, but one, were familiar with *Where's Wally?*TM prior to the start of the task. Verbal instructions to the task were given, nonetheless. Brief instructions ("Search for Wally in his red striped top. Press a key when looking at him") were

also projected onto the screen prior to the start of each trial. The four trials were always presented in the same order. Participants were not specifically instructed as to which direction/side from which to begin.

5.5.2.2 Definitions

The Where's Wally?TM task was made up of four trials; each trial showed an illustration in which many people were depicted at a given location. The last calibration-check target, immediately prior to stimulus presentation, was termed the 'neutral point'.

5.5.2.3 Task-specific calibration check

Immediately prior to the presentation of each trial's stimulus, participants were asked to fixate on six pre-selected targets which were presented one-at-a-time. The targets were distributed to give adequate coverage over an area marginally smaller than the field in which stimuli were presented (36° horizontally by 22° vertically). The targets were presented quasi-randomly; the last target was always the (0°, 0°) target (i.e., the neutral point), while the first five targets appeared in a random order. See Figure 5-24 for an example.



Figure 5-24. The Airport Trial of Where's Wally?TM with overlaid calibration-check targets. The circled target was always the last to be presented (i.e., the neutral point).

5.5.2.4 Protocol

The four Task trials were selected from the Where's Wally?[®] 2012 Calendar (The Ink Group, 2012), and included scenes of a museum, an underground maze and an airport. The choice of the scenes was not arbitrary. The museum scene was shown twice (Trial 1 and Trial 4); this was done to evaluate improvements in the time to finding Wally and/or trial score. The maze scene (Trial 3) depicted people in near horizontal rows in an underground dungeon. Because the scene appeared 'orderly', it was hoped that tracking eye movements may shed some light on any difference in the "search strategy" (i.e., eye movement pattern) between the study groups.

All trials were initiated by the researcher by pressing any key on the main keyboard which led to the presentation of the trial stimulus. A wireless keyboard was provided to participants who were instructed to press any key whilst fixating on Wally once they were confident they found him. This matched the end of the trial. Participants were not told that the task had a time-limit. Each of the Where's Wally?TM trials automatically ended if no answer was provided after three minutes

from the start of the trial. See Figure 5-25 for a diagrammatic representation of the Task protocol.

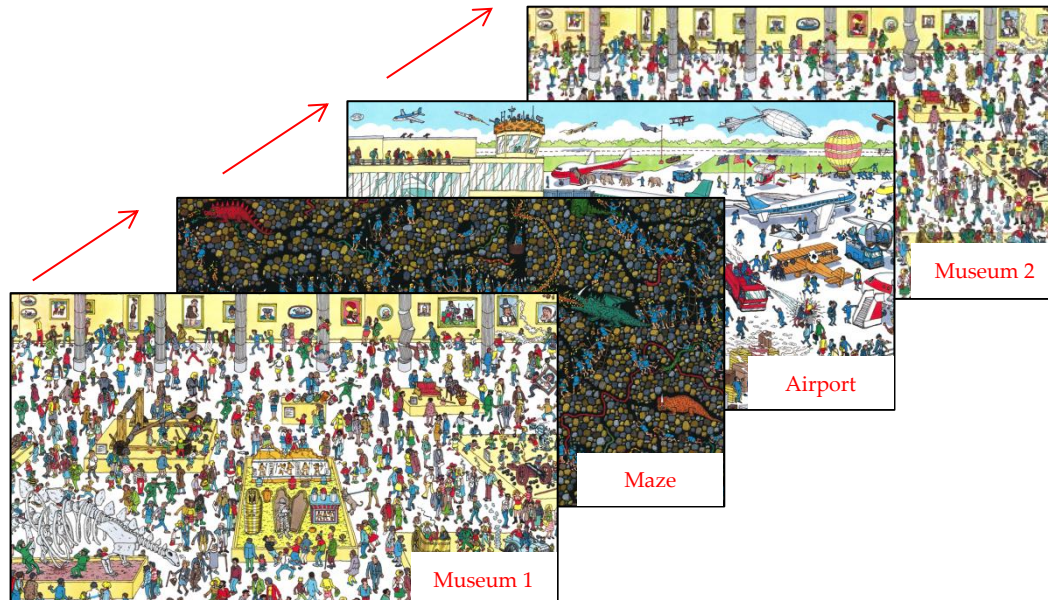


Figure 5-25. A visual representation of the order in which the Where's Wally?TM trials were presented.

5.5.2.5 Correctness

Participants' eye movements were traced on the researcher's screen in real time (visualised as a moving cross atop the background scene). No hints were provided for the participant once trials had started. Participants scored two points for correctly identifying Wally, one point for identifying a red herring character (i.e., Wilma, Wally's doppelgänger friend, or Wenda, her identical twin sister). No points were awarded for incorrect responses or if time ran out.

5.5.2.6 Search strategy and saccade data

Participants' search strategy for each trial was observed. This parameter was quantified by measuring the saccade directions (in degrees), and determining whether the majority were vertical/horizontal (0°, 90°, 180° and 270°) or diagonal.

As mentioned earlier, this was of particular interest in the Maze scene where the objects and people were drawn largely in horizontal paths.

Due to the nature of the task (up to three minutes in duration, large field of search and the small target), it was much more difficult to maintain complete tracking of eye movements compared with other tasks. When tracking was lost, the participant's saccade data would not be recorded. To accommodate this, it was decided to include saccade rate (i.e., saccades per second) whereby the number of recorded saccades was divided by the time during which tracking was available.

Saccade data, rather than fixation data, were thought to be more meaningful to measure. Combined with saccade rate, the saccade amplitude was thought to be reflective of the participant's search strategy. An upper limit of 42.2° (maximum possible amplitude) was applied to saccade amplitude. This provided near-complete coverage of the area in which Where's Wally?™ scenes were presented.

5.5.3 Results

5.5.3.1 Overview

Eleven participants (four NC, three PD-N and four PD-MCI) were unable to complete the Where's Wally?™ task, mainly due to technical complications. This left a total of 38 participants who completed the task (12 NC, 14 PD-N and 12 PD-MCI). Several parameters (see below) were recorded, each of which had quality-assurance criteria. These criteria, considering the nature of the task (large field of search combined with a small target), were relatively stringent; data falling short of these criteria (a sizeable amount compared to other tasks' data) were discarded. Thus, depending upon the parameter's criteria, the total number of participants may not always be equal to the total number of participants completing the task.

5.5.3.2 Duration to trial completion

Trial duration data were available for all participants completing the task ($n = 38$). The average duration taken to complete each of the four Where's Wally?™ trials was calculated for each participant. The per-group mean durations were: 51.7 seconds (± 12.3) for NC, 56.3 seconds (± 9.1) for PD-N and 58.3 seconds (± 25.2) for PD-MCI. There was no statistically significant difference between the three study groups. Only three participants exhausted the three-minute time-limit without providing an answer: two PD-N participants (trial 1 for one and trial 3 for the other) and one PD-MCI participant (trial 1).

To examine for a learning effect, the difference in duration between completing Museum 1 (trial 1) and Museum 2 (trial 4) was calculated. Despite the marked difference in the mean duration difference between NC vs. PD-N vs. PD-MCI (18.5 seconds vs. 11.4 seconds vs. 31.1 seconds, respectively), it did not reach statistical significance due to the heterogeneous performance of individuals within each group (standard deviations of 31.3 seconds, 17.9 seconds and 53.4 seconds, respectively).

5.5.3.3 Correctness

Data pertaining to response correctness were available for 37 participants (12 NC, 14 PD-N and 11 PD-MCI). Each trial in the Where's Wally?™ task was scored out of 2 points (total 8 points). Each participant's aggregate score was converted to a percentage. The mean percentages of correct answers were 30.9% ($\pm 17.2\%$) for NC participants, 24.7% ($\pm 14.8\%$) for PD-N participants and 13.6% ($\pm 13.8\%$) for PD-MCI participants, with a significant difference in between-group performance ($p = 0.036$). Post-hoc analysis showed PD-MCI participants scored significantly lower

than NC participants ($p = 0.011$). Scores of PD-N participants were not significantly different from NC ($p = 0.27$) or PD-MCI ($p = 0.10$).

5.5.3.4 Search strategy and saccade data

Analysis of saccade direction revealed a generally preserved pattern of scene search by NC and PD-N participants; most of the saccades were horizontal, which fits with the general layout of the Maze trial. PD-MCI participants, on the other hand, made more vertical saccades, which appears at odds with the orderly layout of the scene (see Figure 5-26).

To quantify the observed difference, values of saccadic directions—which varied from 0° to 360° —were transformed to be within a single quadrant (i.e., $0-90^\circ$). This allowed meaningful calculations of mean saccade directions with regards to horizontal or vertical tendencies (i.e., larger mean values indicate a higher proportion of vertical saccades). Saccades made by PD-MCI participants deviated significantly more from the horizontal ($39.7^\circ \pm 8.5$) in the Maze trial, compared with NC ($33.2^\circ \pm 3.3$) and PD-N ($31.4^\circ \pm 3.2$) participants ($F_{2, 18} = 4.4$, $p = 0.028$).

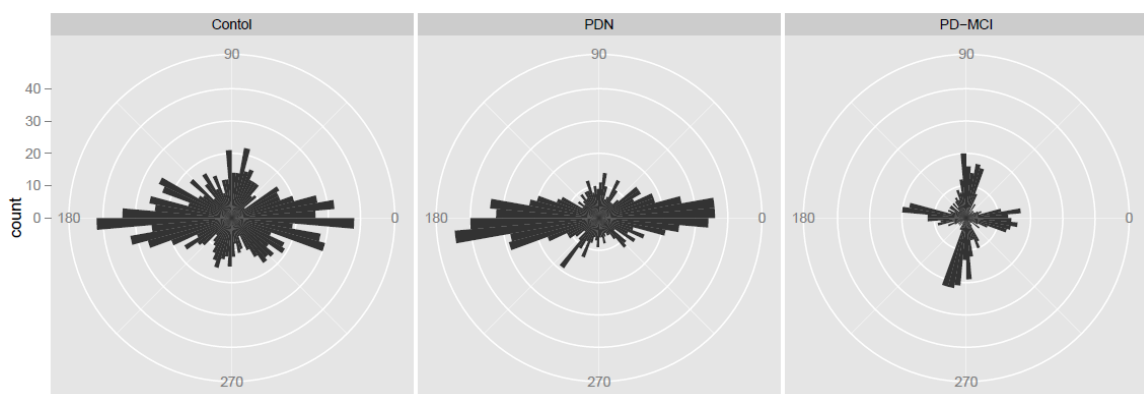


Figure 5-26. Direction of saccades made by participants from each group during the Maze trial.

The mean tracking ratio (i.e., the ratio of summed fixation durations—excludes blinks—divided by the sampling frequency of BeGaze™; expressed in percent) for all 38 participants across the four trials was 84.3% (range, 34-95%). There was no significant difference in the mean saccade rate between the study groups: 3.9 /s (± 0.7) for NC, 4.4 /s (± 2.1) for PD-N and 3.9 /s (± 1.4) for PD-MCI ($p = 0.68$). Moreover, there was no significant difference in the saccade amplitude between NC ($6.6^\circ \pm 1.5$), PD-N ($5.8^\circ \pm 0.9$) and PD-MCI ($5.6^\circ \pm 1.0$), ($p = 0.12$). Saccade peak velocity was similar between the study groups: NC 269.6 °/s (± 55.6), PD-N 245.9 °/s (± 37.3) and PD-MCI 253.2 °/s (± 64.7), ($p = 0.72$).

5.5.4 Discussion

5.5.4.1 Overview

The number of participants for whom complete data were available was substantially lower compared with other tasks. This could be attributed to several reasons. The task was performed towards the end of the first testing session, by which time some participants might have been more prone to distraction and fatigue. Moreover, the field in which the trials were projected was the largest of all tasks' ($36^\circ \times 22^\circ$), and the target (i.e., Wally) was among the smallest targets of all tasks. Finally, the time allowed to complete the task was, in retrospect, too long. The initial intention was, by providing ample time, to avoid pressuring participants to give a hasty answer (i.e., ultimately disturbing the 'natural' search pattern). However, this also led to an increased chance of technical lapses when eye movements could not be recorded. To circumvent this problem, rate of saccades (as opposed to number of saccades) was utilised in an attempt to employ as much of the viable data as possible.

5.5.4.2 Trial duration and correctness

The mean total duration for each trial did not significantly differ between the study groups: NC, 51.7 s (± 12.3); PD-N, 56.3 s (± 9.1); PD-MCI, 58.3 s (± 25.2); $F_{2,35} = 0.5$, $p = 0.61$. As expected, PD-MCI participants scored significantly lower on this task than NC participants; PD-N participants did not differ significantly in their scores from either group.

What is intriguing is the marked, albeit not statistically significant, duration difference between the groups when performing the two Museum trials. PD-MCI participants spent an average of 31.1 seconds less on Museum 2 than on Museum 1. This is compared with 18.5 seconds for NC and 11.4 seconds for PD-N. This may be attributed to one of two explanations: a learning effect or increased distractibility as participants progressed through trials.

To uncover any possible learning effect, group performance on Museum 1 and Museum 2 trials was examined more closely. A learning effect was defined as faster location of Wally or Wilma/Wenda in Museum 2 compared with Museum 1, or improved score (i.e., finding Wally in Museum 2 after not having found him in Museum 1).

Table 5-12. Assessing per-group learning effect based on the difference in their performance in Museum 1 and Museum 2 trials.

Group	N	Duration difference (s)	ANOVA	p	N	Score difference (%)	ANOVA	p
NC	12	- 65.1	$F_{2,35} =$	< 0.001	9	+18.1%	$F_{2,26} = 1.6$	0.001
PD-N	14	- 62.1	0.96	< 0.001	10	+1.3%		0.78
PD-MCI	12	- 51.8		< 0.001	10	+1.2%		0.34

As shown in Table 5-12, only NC participants showed a significant learning effect on score; they located Wally or Wilma/Wenda with more accuracy in Museum 2 compared with Museum 1. PD participants, on the other hand, while completing Museum 2 trial faster, their score did not significantly improve. Therefore, it appears as though NC participants had ‘learnt’ from their mistakes in the Museum 1 trial, whereas PD participants repeated their mistakes.

5.5.4.3 Search strategy and saccade data

There was a stark contrast between the search strategies employed by NC and PD-N, and the strategy of PD-MCI participants (see Figure 5-27 for representative examples of eye movement ‘scan paths’ – the fixation sequences).

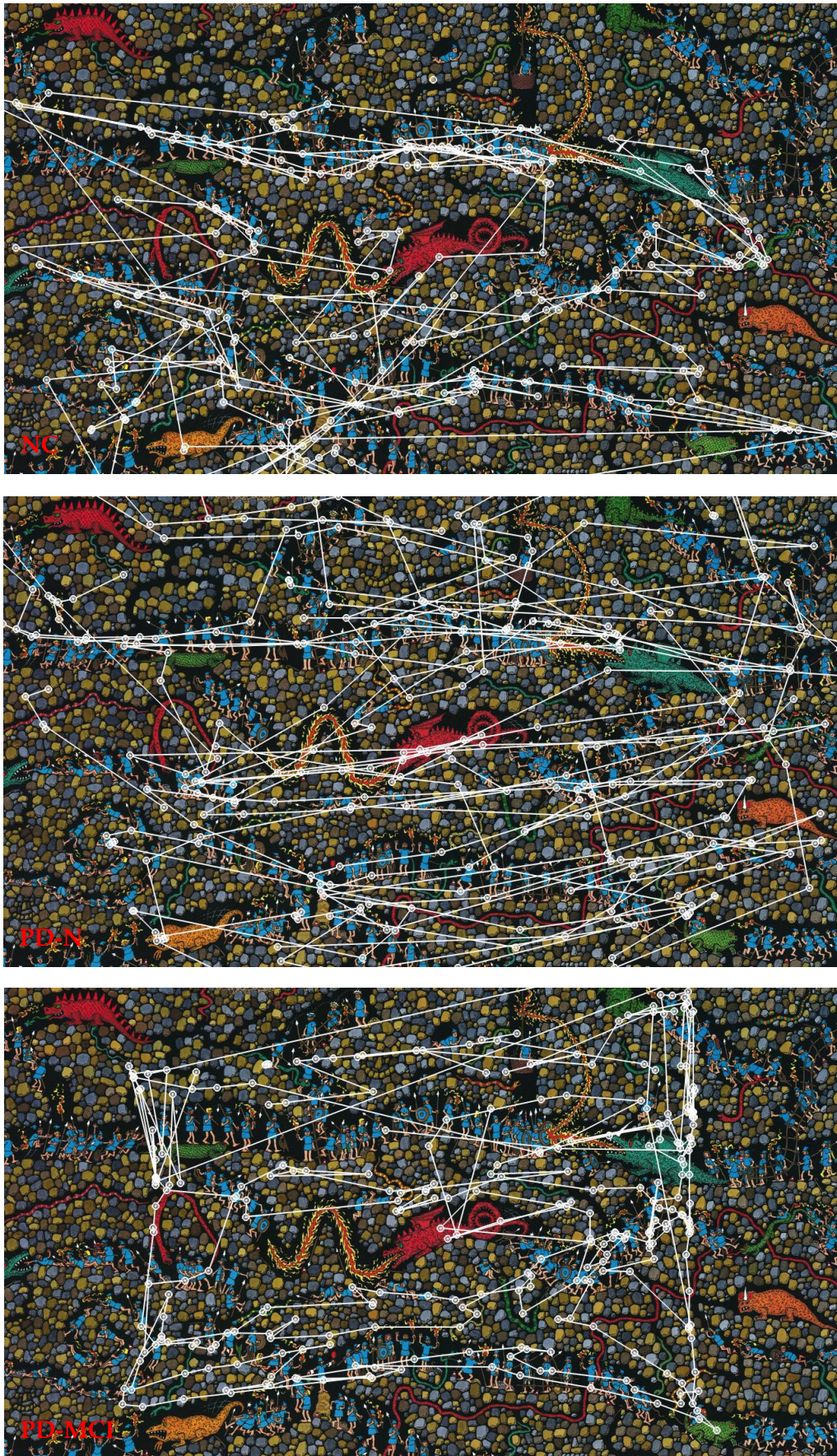


Figure 5-27. Representative scan paths (fixations are represented by circles and saccades are represented by the connecting lines) of three participants: one from the NC group (top), one from the PD-N group

(middle) and one from the PD-MCI group (bottom). Note the limited scanning area as well as the different search strategy (i.e., more vertical saccades) employed by the PD-MCI participant compared with the NC and PD-N participants.

Several factors have been described in the literature as influencers of eye movement direction. The effect of cognitive status, however, does not appear to have been addressed before. To understand how a person's cognitive status affects the pattern of their eye movements, other factors must be addressed first; these will be discussed from most general to more specific.

At the most basic level are biological and evolutionary factors. The human head (through motion at the cervical spine) moves more horizontally (i.e., rotation, to almost 180°) than it does vertically (i.e., flexion/extension, up to 160°), (Windle, 1980). These movements substantially enhance the visual field of humans, whose two horizontally-arranged eyes are much more restricted vertically (around 90°) than horizontally (up to 180°) (Pelz, Hayhoe, & Loeber, 2001; Smeets, Hayhoe, & Ballard, 1996). Furthermore, Collewyn and colleagues (1988) have shown that vertical and oblique saccades are slower than horizontal saccades, and Becker *et al* demonstrated that vertical saccades are more curved than horizontal saccades (1990). It may well be that, over time, we learn to favour horizontal over vertical saccades (Petit et al., 1996), as they allow us to maximise the gain (i.e., details seen) for a minimum cost (i.e., less time and fewer saccades). This, however, may be influenced by other factors described next.

More specific to groups of people are cultural and experiential factors pertaining to specific tasks. When presented with non-directional visual patterns (e.g., dots, circles or bars), Middle Eastern subjects have shown preference (Abed, 1991) for and higher proficiency (Nachshon, Shefler, & Samocha, 1977) in right-to-left scanning (since Arabic and Hebrew are read right-to-left). Western subjects were more likely to show the opposite pattern. East Asian subjects, who have

traditionally read vertically, going from right to left, demonstrated a 1:1 ratio of horizontal to vertical saccades. The latter finding is in contradiction to the 2:1 ratio observed in Middle Eastern and Western subjects.

Finally, scene-specific factors need to be considered. The rectangular screen onto which stimuli from the Behavioural Markers study were projected is wider than it is high. Moreover, people depicted in the particular Where's Wally?TM stimulus chosen (i.e., the Maze task) were arranged in horizontal rows. Therefore, more information was available on the right and left of the scene (as opposed to above and below). Only NC and PD-N participants, however, seem to have taken advantage of this.

Taken together, these factors provide a rationale for the bias for horizontal over vertical saccades. Since more specific factors (i.e., stimulus-display and cultural factors) were the same across the participants, this leaves biological factors as the likely explanation of the peculiar pattern of eye movements of PD-MCI participants.

The direction of microsaccades (which are generally involuntary and horizontal in humans; Cui, Wilke, Logothetis, Leopold, & Liang, 2009; Otero-Millan et al., 2011) has been previously shown to be affected by the person's attention (Engbert, 2006; Engbert & Kliegl, 2003) and working memory (Otero-Millan et al., 2008; Valsecchi & Turatto, 2009). Moreover, Kapoula and colleagues (2014) have recently shown that microsaccades deviated from the horizontal in patients with AD and amnesic MCI. The authors point the differences detected in microsaccade direction could be related to attentional deficits present in AD (Perry & Hodges, 1999; Riva et al., 1999) and MCI patients (Belleville, Chertkow, & Gauthier, 2007; Levinoff, Saumier, & Chertkow, 2005). They acknowledge, nonetheless, that further research is

required to see if changes in microsaccade direction are associated with specific attentional deficiencies (Kapoula et al., 2014).

Several differences between Kapoula *et al's* study (2014) and the present behavioural markers study exist. The studied populations (AD vs. PD), nature of involved task (fixating on a stationary cross vs. a search task) and types of eye movements (microsaccades vs. reflexive saccades) are all different. Yet the 'abnormal' pattern of eye movements displayed by both MCI—amnesic MCI and PD-MCI— groups (assuming microsaccades and saccades are parts of a continuum; Martinez-Conde, Macknik, Troncoso, & Hubel, 2009) is similar; MCI participants' eyes move up and down more than eyes of humans with normal cognition. Further research to clarify (or dispel) any relationship between our findings and those of Kapoula *et al's* (2014) is axiomatic.

Despite dissimilar search strategies, performance in the Maze trial was not significantly different between the groups. Neither the saccade rate ($F_{2, 24} = 1.1, p = 0.36$) nor trial duration ($F_{2, 24} = 1.6, p = 0.23$) was found to be significantly different between NC, PD-N and PD-MCI participants. Furthermore, the Maze trial-specific score was not significantly different between the study groups. This latter variable, however, is probably less likely to be meaningful due to a floor effect. Only three participants (two NC and one PD-MCI) were able to locate Wally.

5.6 Jeddah map search task

5.6.1 Background

In this task, the subject is shown a map of Jeddah, Saudi Arabia. In it are several copies of different symbols (e.g., petrol pump symbol signifying a petrol service

station). The subject is instructed to locate as many of the petrol station symbols as they can. This is aimed to examine visual selective attention. In essence, this task is similar to the familiar Map Search task of the Test of Everyday Attention (TEA; Robertson, Ward, Ridgeway, & Nimmo-Smith, 1996).

Among neurological populations, map search tasks have been best described in traumatic brain injury (TBI) patients, followed by stroke patients (Lezak, Howieson, Bigler, & Tranel, 2012; pp. 426). Performance on the Map search and Telephone search subsets (of the Test of Everyday Attention) was the most sensitive indicator in differentiating moderate-to-severe TBI patients from normal controls (Bate, Mathias, & Crawford, 2001; Chan, 2000). Given the relative scarcity of data on map search task performance by PD patients, the Jeddah Map Search task was included in order to assess this; simultaneous recording of eye movements was hypothesised to enrich the data gathered by providing details on the strategy utilised by the searching participant.

5.6.2 Methods

5.6.2.1 Overview

None of the participants was familiar with Jeddah's geography. Participants were asked to look for petrol stations. The corresponding symbol (see Figure 5-28) was magnified and shown to participants prior to starting the task. Brief instructions ("Search for petrol stations. Press a key when looking at each one, and say when you have finished") were also projected onto the screen prior to the start of the task. Participants were not told the total number of petrol stations shown in the map, nor were they instructed as to the direction/side from which to begin.

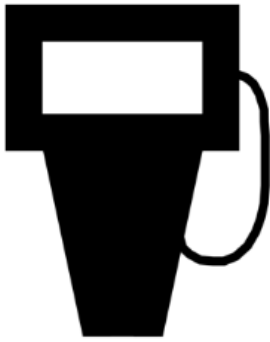


Figure 5-28. The petrol station symbol as found on the Jeddah map.

5.6.2.2 Definitions

The Jeddah Map Search task was made up of three identical trials. The last calibration-check target, immediately prior to stimulus presentation, was termed the 'neutral point'.

5.6.2.3 Task-specific calibration check

Immediately preceding the presentation of each trial's stimulus, participants were asked to fixate on six pre-selected targets which were presented one-at-a-time. The targets were distributed to give adequate coverage over an area marginally smaller than the field in which stimuli were presented (20° horizontally by 17° vertically). The targets were presented quasi-randomly; the last target was always the (0° , 9°) target (i.e., the neutral point), while the first five targets appeared at no particular order. See Figure 5-29 for an example.

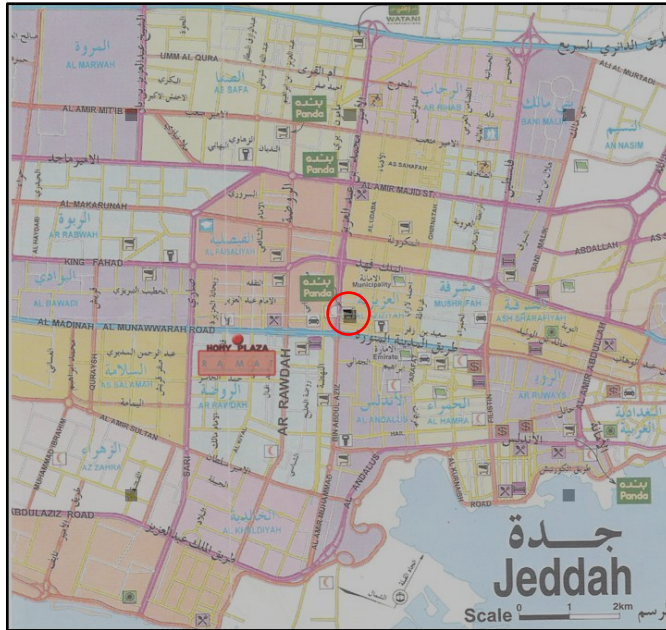


Figure 5-29. The Jeddah Map Search task, with overlaid calibration-check targets. The circled target was always the last to be presented (i.e., the neutral point).

5.6.2.4 Protocol

A freely available map of the Southwestern part of the researcher's hometown in Saudi Arabia, Jeddah, was chosen for the last task. This visually cluttered map contained the names of streets and suburbs in both Arabic and English, as well as symbols for the various amenities (e.g., banks, hospitals, petrol stations, restaurants and supermarkets). Participants were asked to localise the petrol stations, of which there were six.

All trials were initiated by the researcher by pressing a key on the main keyboard which led to the presentation of the trial stimulus. A wireless keyboard was provided to participants who were instructed to press any key whilst fixating on the petrol station symbol once they were confident they found one. The trial was ended by the researcher upon the participant reporting that they could not locate any more petrol stations.

5.6.2.5 Correctness

Participants' eye movements were traced on the researcher's screen in real time. No hints were provided for the participant once trials had started. Participants scored one point for correctly identifying a petrol station. No points were awarded for missed petrol stations. Participants were not penalised for incorrect responses.

5.6.2.6 Trial duration

The time the trial ended was recorded by BeGaze™. A mean duration (across the three trials) was calculated for each participant.

5.6.2.7 Saccade data

The Jeddah Map Search task was, in some ways, similar to Where's Wally?™ in that participants had to search a large field for small targets. Therefore, it was more difficult to maintain complete tracking of eye movements compared with other tasks. When tracking was lost, the participant's saccade data were not recorded. To accommodate this, it was decided to include saccade rate (i.e., saccades per second) whereby the number of recorded saccades was divided by the time during which tracking was available.

Saccade data, rather than fixation data, were thought to be more meaningful to measure. Combined with saccade rate, the saccade amplitude was thought to be reflective of the participant's search strategy. An upper limit of 26.2° (maximum possible amplitude) was applied to saccade amplitude. This provided near-complete coverage of the area in which Jeddah Map Search scenes were presented.

5.6.3 Results

5.6.3.1 Overview

Acceptable data were available for a total of 38 participants: 12 NC, 14 PD-N and 12 PD-MCI. Physical fatigue and technical difficulties were the two most common reasons data were not collected from ten participants. Several parameters (see below) were recorded, each of which had certain quality-assurance criteria. Participants' data falling short of these criteria were discarded. Therefore, depending upon the parameter's criteria, the total number of participants may not always be equal to the total number of participants completing the task.

5.6.3.2 Correctness and trial duration

In general, NC participants had the highest mean of correct responses and shortest mean of trial duration, compared with PD-N and PD-MCI participants. The differences between the means, however, were not statistically significant (see Table 5-13). In addition, mean aggregate scores for trial 1 (2.6 points \pm 1.3), trial 2 (3.0 points \pm 1.2) and trial 3 (2.9 points \pm 1.6) were not significantly different ($F_{2, 71} = 0.64, p = 0.53$).

Table 5-13. Comparing trial scores and durations of the study groups.

	Correctness			Trial duration		
	NC	PD-N	PD-MCI	NC	PD-N	PD-MCI
N	10	13	8	12	14	12
Mean	3.2 pts	2.5 pts	2.2 pts	34.6 s	36.6 s	53.0 s
SD	1.3 pts	1.1 pts	1.0 pts	13.1 s	17.5 s	30.0 s
ANOVA	F _{2, 28} = 1.8			F _{2, 35} = 2.8		
p	0.18			0.077		
Pts = points; s = seconds						

5.6.3.3 Saccade data

The mean tracking ratio across the three Jeddah Map Search trials was 76.4% (range, 24.5-95.3%). There was no significant difference in the mean saccade rate between NC ($4.4 /s \pm 2.4$), PD-N ($3.9 /s \pm 1.5$) and PD-MCI ($4.2 /s \pm 2.2$), ($F_{2, 28} = 0.19$, $p = 0.83$). Furthermore, there was no statistically significant difference in the saccade amplitude between the study groups: $5.0^\circ (\pm 1.6)$ for NC, $4.6^\circ (\pm 0.5)$ for PD-N and $4.3^\circ (\pm 0.4)$ for PD-MCI ($F_{2, 28} = 1.1$, $p = 0.35$).

5.6.4 Discussion

5.6.4.1 Overview

The number of participants for whom complete data were available was acceptable, albeit less than most other tasks. The chosen map of Jeddah, although conceptually mimicking TEA's Map search task (Robertson et al., 1996), has no standardised performance normative data. The objective of this task was to compare performance on a search task between the three study groups, acknowledging external applicability may be limited.

5.6.4.2 Correctness and trial duration

Echoing results of other tasks in the behavioural markers study, PD-MCI participants trended, albeit non-significantly, towards a worse performance and a longer period of time for task completion. Perhaps the addition of a PD-D group would have resulted in a significant difference between the study groups; this remains a speculation, however.

Whilst general improvement in the performance of all participants across the three trials was expected, this was not observed. This could be due to a floor effect, since none of the participants was able to locate all six stations. Moreover, because participants were not given feedback whether their answers were correct or not, this may have led some participants to locate six targets but not all of which were correct. The latter explanation is less likely given the fact that incorrect responses were mostly random targets that differed from the petrol station symbol in shape, and sometime colour.

5.6.4.3 Saccade data

Significant between-group differences in saccade rate and saccade amplitude were lacking. This is akin to findings from the Where's Wally?TM task described previously.

5.7 Chapter summary

This component of the behavioural markers study was conducted using bench-mounted laboratory eye tracking. In the MoCA animal identification task, PD-MCI participants were significantly slower to fixate on the first animal of Trial₁ ($p = 0.002$) than NC and PD-N participants. The latency to fixating on the first animal of Trial₂ by PD-MCI participants was significantly shorter ($p = 0.016$) than Trial₁ – indicating a learning effect. This was not observed in Trial₃ in PD-MCI participants, or in any of the trials by NC and PD-N participants. In addition, PD-MCI participants scored significantly lower than NC and PD-N participants on the Where's Wally?TM search tasks ($p = 0.036$).

The overall results of the laboratory-based eye movement tasks were largely similar among the three study groups, however. It is vital to note that PD-MCI participants included in this study were selected from a cohort of PD patients who in fact fulfilled the diagnostic criteria of *mild* cognitive impairment as proposed by Litvan *et al* (2012). Inadequate screening of research participants (e.g., just using the MMSE) may lead to the potential inclusion of PD-D patients masquerading as PD-MCI patients (Fernandez, Crucian, Okun, Price, & Bowers, 2005). This, in turn, may exaggerate findings on neuropsychological tests that are otherwise milder or absent in PD-MCI participants.

The addition of a PD-D group to this study will give a fuller picture of the association between eye movements and performance on neuropsychological tests in this disease. Based upon the above findings, it may be the case that PD-MCI participants compensate in their performance, only to deteriorate rapidly once they reach the PD-D stage.

CHAPTER 6

Dynamic Eye Movement Tasks

6.1 *Introductory remarks*

6.1.1 Study aims and hypotheses

Dynamic tracking (i.e., using mobile apparatus) of eye movement abnormalities in PD patients—albeit technically more cumbersome and less robust—may in fact be more clinically relevant than static laboratory-based measurements. In addition, dynamic eye movement-tracking allows a closer investigation of eye movement abnormalities during ambulation (Lohnes & Earhart, 2011). The risk of falls in PD patients, especially those with gait freezing, is several-fold higher than in healthy age-matched controls (Pickering et al., 2007). Insights gained from eye movement analysis, for example during the performance of different types of daily activities, may aid in uncovering strategies to mitigate the significant morbidity and mortality associated with falls in patients with PD.

Since measuring eye movement dynamics is objective, fast and non-invasive, we sought to examine the differences in eye movement behaviour in PD patients vs. healthy controls during the performance of selected neuropsychological and natural tasks. Study hypotheses have been described in the previous chapter.

6.1.2 Laboratory settings

6.1.2.1 *Setting in the MoVELab*

Participants were seated on a desk chair with no arm rests, in front of a standard 120 x 59 cm office desk. Tasks were presented to participants in paper-form, resting on a wooden board angled at 60°. The angled board was placed on the desk, centred horizontally, with the front of the angled board approximately 5 cm from the edge of the desk (see Figure 6-1). To accommodate for variations in participant heights, VA

and comfortable reading distances, participants could select their own seat location; most participants, however, positioned the chair approximately an arm's length away from the angled board.

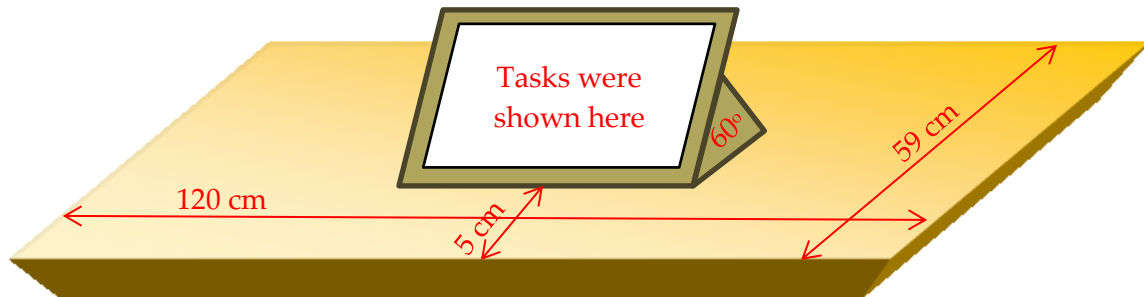


Figure 6-1. Desk setting for the MoVELab tasks, which were presented to participants on the angled wooden board (illustration adapted with permission from Toh, 2016).

Once familiarised with the laboratory setting, participants were fitted with the modified baseball cap, fitted with the two cameras of the iViewX™ HED system as described earlier. Then, participants were again allowed time to acquaint themselves with the head-mounted eye-tracker system prior to the onset of any tasks.

Recordings from the left eye (i.e., tracking the left pupil) were the default. Recordings from the right eye, however, were necessary for four participants in whom adequate recordings from the left eye could not be obtained, despite multiple adjustments and calibration attempts. The iViewX™ HED system allows for recordings from either eye with no compromise of data quality (SMI, 2010). Participants who could not adequately visualise the tasks without spectacles were excluded, as these interfered with proper tracking of the pupil.

6.1.2.2 Calibration protocol

After adequate positioning of the participant, and prior to task commencement, a calibration procedure was undertaken. This comprised a manual process in which the eye-tracker was made to match raw eye-position data from each participant relative to fixation positions in the environment. Once seated in front of the desk, each participant was asked to fixate on verbally specified nine pre-selected targets printed on an A3-sized paper (as shown in Figure 6-2). The same calibration-check was constantly reviewed, and repeated if necessary, between tasks. Once satisfactory tracking was achieved, the calibration sheet was manually lifted by the researcher from the top left corner, revealing the task paper underneath.

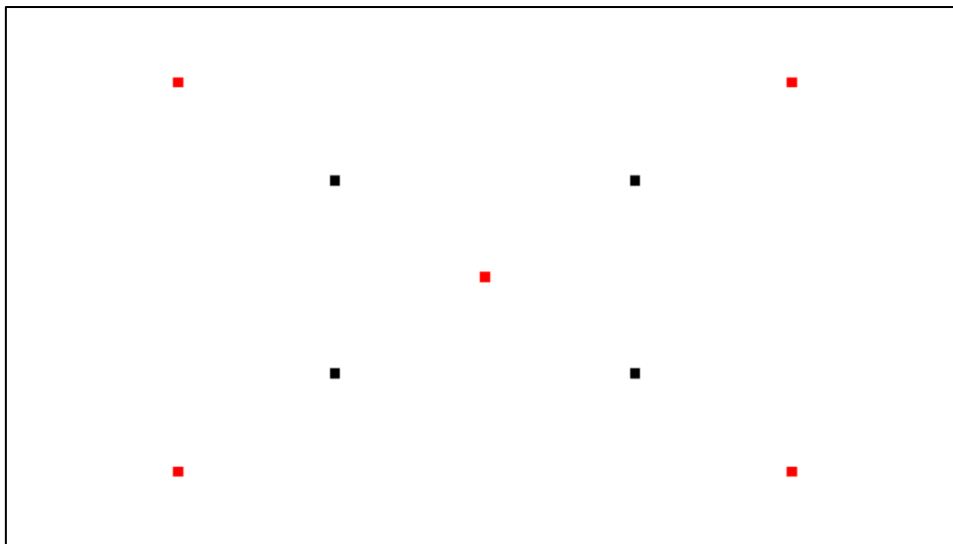


Figure 6-2. Calibration targets for the dynamic eye movement tasks.

6.1.2.3 BeGaze™ settings

Automated data on saccadic eye movements were computed by the BeGaze™ software (SMI, 2013) offline. During analysis, fixations with duration less than 50 ms or greater than 1 500 ms were excluded. Saccades with amplitude greater than 25°, or duration greater than 150 ms were also excluded. Saccades were filtered to include only those with start and end locations within the pixel dimensions of the scene video

(752 × 480). Therefore, saccades with a start or end locations less than zero, or greater than 752 or 480 for X and Y locations, respectively, were excluded. Saccades with locations outside these values represented instances in which eye tracking was lost at the start or end of the saccade, producing eye movement data that were invalid. Whilst several participants had low tracking rates, their data were included as valid information about measures, such as saccade amplitude and fixation duration, were still attainable from the period in which valid tracking occurred.

Inability of the iViewX™ HED system to maintain tracking can occur due to a number of reasons, such as a high rate of blinking or ptosis of the eyelid of the tracked eye. Such conditions obstruct the camera's view of the pupil, which is used to calculate the position of the eye. Participants with a high amount of lost tracking were excluded from the task-specific analysis in order to avoid analysing inaccurate fixation patterns.

6.1.3 Study sample

From an initial sample of 48 participants for the behavioural markers study, only 37 participants were included in the dynamic eye tracking part. The most common reason for exclusion was the need to wear spectacles or contact lenses during task performance (n = 8); other reasons included persistent poor quality of data collected in previous tasks (n = 1) and refusal to continue due to physical and mental fatigue (n = 2). The remaining sample was made up of 13 NC participants, 12 PD-N participants and 12 PD-MCI participants. No significant changes to the previously described sample characteristics were observed.

6.2 *SDMT written task*

6.2.1 Background

With the help of eye-movement tracking techniques, insights into participants' oculomotor scanning patterns and visuomotor coordination may be gained. Additionally, I hypothesised that I would be able to capture more of any improvement in task performance (as assessed by trial-scores) upon repeating the task twice. Finally, tracking the participant's eye movements can also allow for a deeper analysis of the quality of and the pattern behind a response; data that are not obtainable when using the oral version.

6.2.2 Methods

6.2.2.1 *Instructions*

The instructions were given verbally before the start of the task. Participants were specifically instructed to start with the top left box and proceed horizontally (i.e., left-to-right) without skipping any boxes; once a row was completed, the participant would move on to the next row and so forth. Participants were not told that the task had a time-limit. However, only the first 90 s of each video were included in subsequent analyses, as this is the specified limit for the task (Smith, 1982).

6.2.2.2 *Apparatus*

The iViewX™ HED eye-tracking system has been described in earlier sections. As stated previously, a red plus-sign was superimposed on the scene video to indicate gaze position in each video frame (see Figure 6-3).

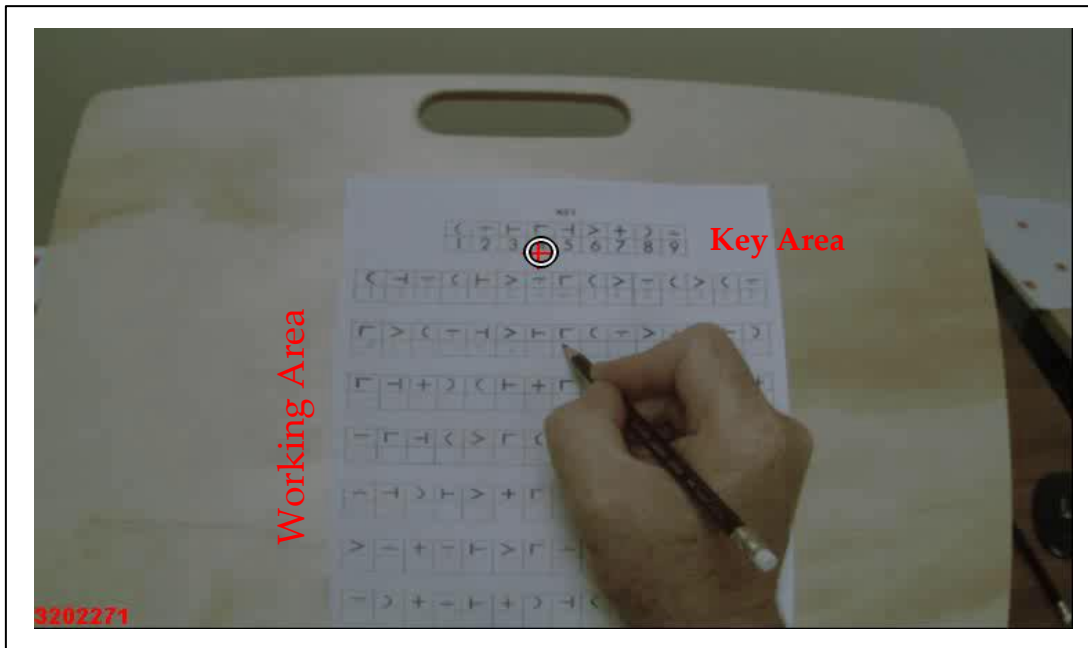


Figure 6-3. Scene video from the head-mounted eye-tracker, showing a participant's gaze position (red plus-sign) whilst completing the SDMT. Here, gaze is directed at the number in the Key below the symbol the participant is currently attempting to match. The participant is about to write the number associated with the given symbol in the Working Area.

The output data was a scene video in which the objects in view could change location freely with the movements of the participant's head. Hence, each fixation from each participant's scene video were mapped onto a static reference image of the SDMT form, in order to aggregate the data. Using the BeGaze™ software, locations of fixations on the SDMT paper were determined by manually mapping the specific location of the gaze cursor to the corresponding location on the reference image. This was carried out in BeGaze™ by referring to the gaze location on the scene video and using a mouse to click on the location of the given fixation on the SDMT reference image. The iViewX™ HD scene video had a resolution of 752 x 480 pixels, whereas the reference image of the SDMT paper had a resolution of 1 240 x 1 753 pixels. Therefore, fixations were transferred from the landscape-orientated scene video to the portrait-orientated reference image (see Figure 6-4).

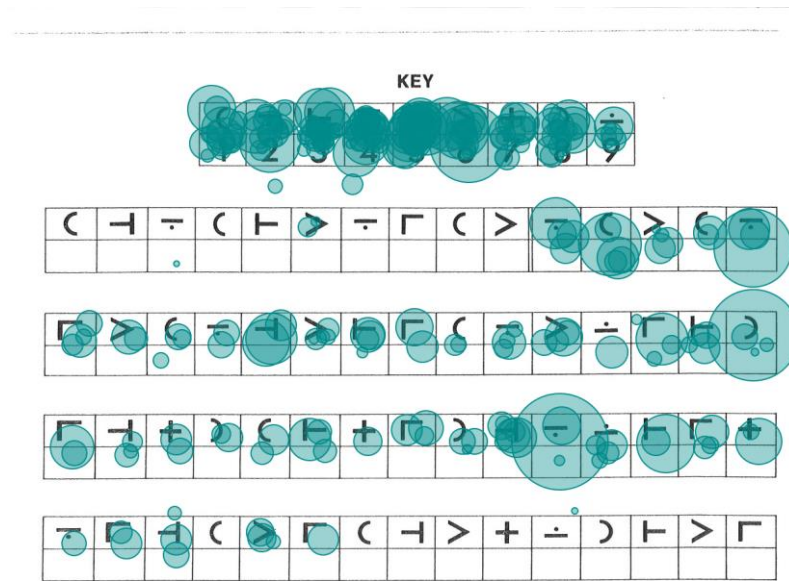


Figure 6-4. Fixation position image generated in BeGaze™ for one of the participants. Circles indicate the locations of fixations, and the diameter of each circle indicates the fixation duration. Fixations during the practice phase (i.e., the first 10 boxes) were excluded. Image credit: Ms Maddie Pascoe.

6.2.2.3 Definitions and task protocol

The SDMT written task was made up of two identical trials: SDMT₁ and SDMT₂. A period of at least 15 minutes was always allowed between the two trials (see Figure 6-5). During this period, participants were made to take a break and engage in unrelated conversation in order to minimise mental rehearsal of the SDMT task. Only data from the SDMT₁ trial were used for eye movement analyses. One point was allocated to each correctly identified symbol within the 90 s time-frame. Because the effect of wearing the head-mounted eye tracker on task performance was unknown, participants' scores in the present study could not be directly compared with standard scores.

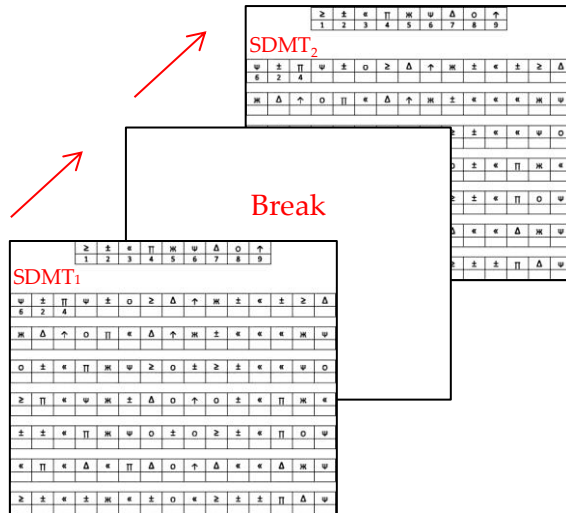


Figure 6-5. A visual representation of the order in which the SDMT trials were presented. In the intervening time between the two trials, participants engaged in other tasks(s) over a period of ≥ 15 minutes (adapted SDMT version from Benedict et al., 2012).

6.2.3 Results

6.2.3.1 SDMT scores

Scores for the SDMT₁ task ($F_{2,35} = 4.0$) were significantly lower in PD-MCI patients (25.8 points \pm 4.9) compared with NC participants (35.4 points \pm 11.5, $p = 0.01$) and PD-N patients (33.4 points \pm 7.5, $p = 0.04$). Significant score improvements between the two SDMT trials were observed in NC ($t_{12} = -5.2$, $p < 0.001$), PD-N ($t_{11} = -5.1$, $p = 0.001$) and PD-MCI participants ($t_9 = -4.5$, $p = 0.017$). The magnitude of the effect is illustrated in Figure 6-6.

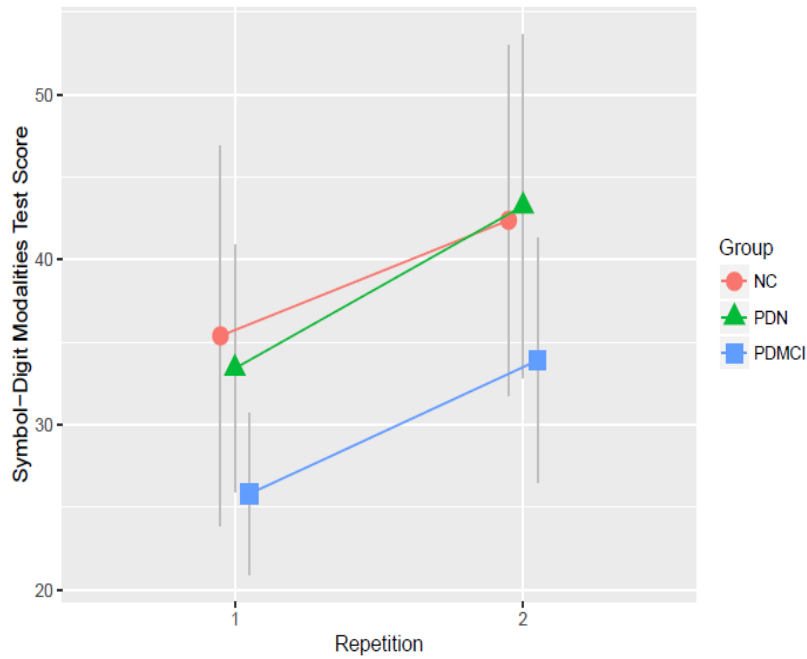


Figure 6-6. The per-group score improvement between SDMT₁ and SDMT₂ trials: NC participants' scores improved by a mean of 7 points, PD-N participants' by 9.8 points and PD-MCI participants' by 8.9 points. Error bars represent one standard deviation above and below the group mean.

6.2.3.2 Correlations between SDMT scores and participant demographics

No difference was found between male vs. female participants. Age was significantly negatively associated with SDMT scores ($r = -0.55$, $p < 0.001$; see Figure 6-7), whereas education was significantly positively associated with the scores ($r = 0.45$, $p < 0.001$). For PD patients, SDMT scores were correlated with UPDRS-III scores ($r = -0.47$, $p = 0.02$; see Figure 6-8) but not disease duration ($r = 0.21$, $p = 0.34$).

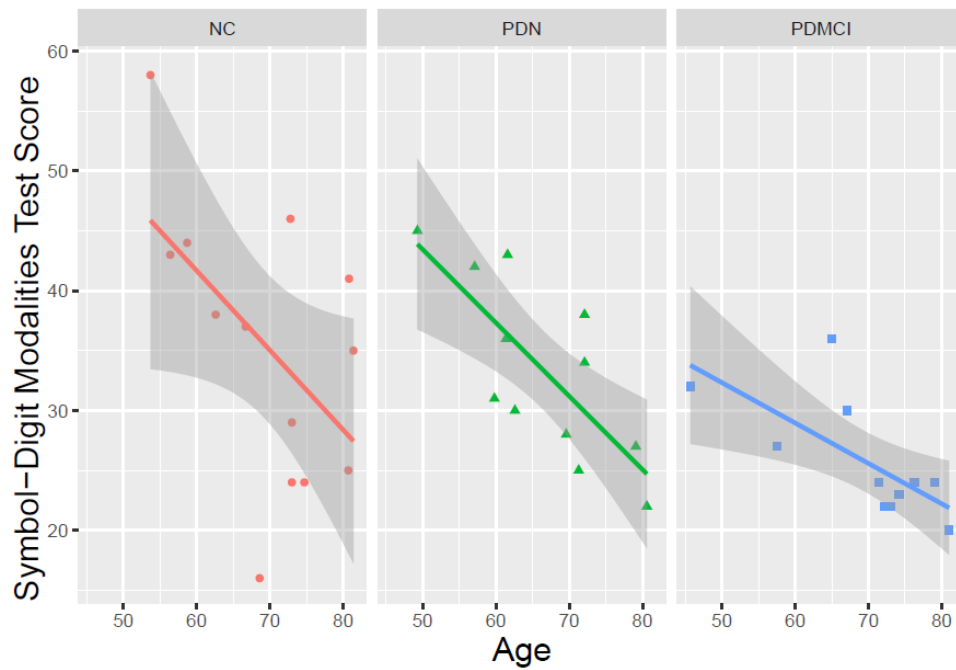


Figure 6-7. The overall significant negative correlation between age and mean SDMT scores was also found within each group.

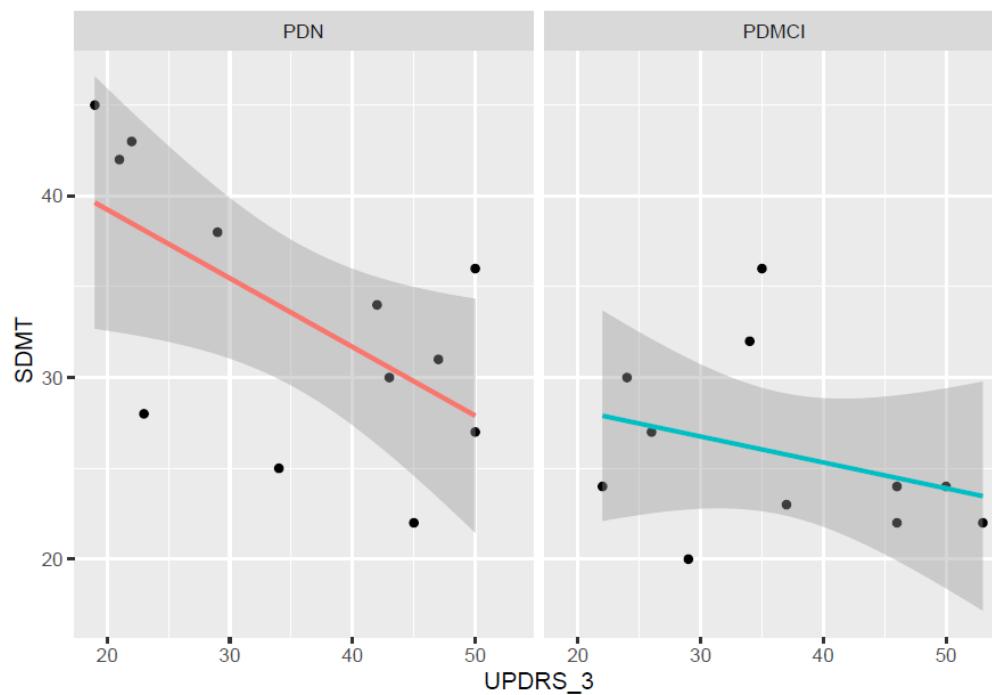


Figure 6-8. There was an overall negative correlation between UPDRS-III scores and SDMT scores. When examined within groups, the correlation was not pronounced in the PD-MCI group.

6.2.3.3 Eye movement parameters

The mean fixation duration was similar among the three groups: 287 ms \pm 77 for NC, 326 ms \pm 75 for PD-N and 289 ms \pm 52 for PD-MCI. Saccade amplitudes (NC, 5.3° \pm 2; PD-N, 3.9° \pm 1; PD-MCI, 4.7° \pm 2.2) did not differ significantly ($F_{2,33} = 2.1$, $p = 0.14$). The mean number of fixations did not differ significantly between the study groups: NC, 226.1 fixations \pm 58.1; PD-N, 227.7 fixations \pm 39.9; PD-MCI 212.1 fixations \pm 63.9 ($F_{2,33} = 0.28$, $p = 0.76$). Secondary analyses of location-specific (i.e., Key vs. Working Areas) fixation numbers also yielded no significant between-group differences.

6.2.3.4 Correlations between mean SDMT scores and eye movement data

Scores on the SDMT were not correlated with the proportion of fixations on the Key Area to the Working Area ($r = 0.2$, $p = 0.28$). Additionally, no significant correlation was found between the SDMT score and mean fixation duration in the Key Area ($r = 0.1$, $p = 0.4$) or the Working Area ($r = 0.1$, $p = 0.4$).

6.2.4 Discussion

6.2.4.1 SDMT performance

The present study investigated the performance of PD participants and matched controls on the SDMT while recording eye movements. PD-MCI participants scored significantly lower than NC and PD-N participants. Repetition of the task, however, resulted in significant score improvements in all three study groups. Eye-movement parameters were similar among the study participants, and were not correlated with task performance.

Age and education were found to be significant predictors of performance on the SDMT, in accordance with a number of previous findings from healthy (Kiely, Butterworth, Watson, & Wooden, 2014) and old MS populations (Charvet, Beekman, Amadiume, Belman, & Krupp, 2014; Van Schependom et al., 2014).

The mean SDMT score of the PD-N group was similar to that of the NC group, indicating comparable performance on the SDMT, despite PD-related motor deficits. This may support the use of the written SDMT *early* in the course of the disease in patients with PD. Among PD participants, higher UPDRS-III scores were associated with significantly lower SDMT scores—a finding which could favour the use of the verbal SDMT in patients with advanced motor symptoms. However, even with similar UPDRS-III scores, PD-MCI participants scored significantly lower on the SDMT compared with PD-N participants (see Figure 6-8). This emphasises the contribution of cognition—in addition to the general motor ability—to the deficit evident in the PD-MCI group, and corroborates previous findings (Beatty, Staton, Weir, Monson, & Whitaker, 1989).

6.2.4.2 SDMT repetition and score improvement

Performance on the SDMT significantly improved—as defined by an increase in the trial-score—in participants from all study groups. With the novel task design (i.e., employing eye movement tracking), we propose an explanation underlying this improvement. We posit that the Key is used as ‘external memory’ during the SDMT. Rather than expending cognitive energy on learning the symbol-digit pairs, participants appear to utilise their ability to use rapid eye movements to seek information. We speculate that upon task repetition, participants’ fixations within the Key Area became more efficient (i.e., faster to locate symbols within the Key), potentially leading to faster completion of task rows with negligible effect on accuracy. This fits in part with findings by Denney and colleagues on a computerised

version of the SDMT (2015). Working memory could be employed to learn symbol-digit pairs during the test, but perhaps due to the time-constrained nature of the test, saccadic eye movements to the Key Area appear to be favoured as the optimal strategy.

6.2.4.3 Eye movement parameters

Despite strong differences in task performance, analysis of eye movement data yielded few differences in eye movement measures between the groups. Our eye movement findings are different to those reported by Elahipanah and colleagues in a group of patients with schizophrenia performing the SDMT (2011). Compared with controls, schizophrenia patients were observed to make more visits to the Key Area per response, and to spend more time in the Key Area per visit (Elahipanah *et al.*, 2011). Direct comparison between the two studies, however, may be problematic due to two caveats; the pathologies studied (PD vs. schizophrenia) involve distinct pathophysiological underpinnings. Moreover, Elahipanah *et al* used a computerised verbal version of the SDMT in which completed responses were immediately obscured—therefore, participants could only refer to the Key Area for guidance on symbol-digit pairings (2011). In contrast, there was nothing to prevent participants in our study from looking at previously completed symbol-digit pairs—rather than the Key Area—to retrieve the correct response.

6.2.4.4 Limitations and future research

Participants were required to wear the head-mounted eye tracker which may have caused distraction, thus impairing performance—although NC and PD-N participants showed similar performance, which may argue against PD participants being differentially impacted by such influence. The contribution of other factors, such as peripheral vision, on the performance of participants could not be

ascertained. Hence, data on the SDMT repetition task must be interpreted with caution. More detailed eye movement analyses, perhaps in conjunction with both the written and verbal SDMT, may provide answers to uncertainties raised by the present study.

6.3 *Natural task: tea making*

6.3.1 Background

6.3.1.1 *Domestic tasks*

In performing day-to-day tasks, human eye movements require precise coordination with, for example, hand movements. In other words, such domestic activities are carried out as a series of visually-guided actions, orchestrated together by a higher-level representation of the task in hand (i.e., a mental “script”; Land, Mennie, & Rusted, 1999).

6.3.1.2 *Tea-making*

Eye movements of human subjects preparing cups of tea were initially intensively studied by Michael Land—a fittingly British neurobiologist. With colleagues, he examined the pattern of task completion in a number of subjects. The resultant patterns were highly consistent among the studies’ subjects (Land et al., 1999). Human eyes deal with one object at a time, with the time spent on each object approximating that of the duration of object manipulation; a number of fixations occur on different parts of the manipulated object. Second, saccadic eye movements were almost exclusive to task-relevant objects, despite a plethora of other objects in the immediate environment.

6.3.1.3 The role of eye movements

As demonstrated by Land *et al* (1999), healthy subjects tend to fixate on objects prior to manually operating them. Our group has previously shown that—in pure oculomotor laboratory-based tasks—PD patients exhibit anticipatory saccades relative to controls; particularly in response to multi-step rhythmical sequences (M. R. MacAskill *et al.*, 2012). This is postulated to be a compensatory mechanism for the hypometric saccades in PD. No study to date has examined eye-hand coordination in PD participants completing a natural task. We sought to examine if the anticipatory asynchrony between fixations and hand actions is preserved in our PD participants.

6.3.2 Methods

6.3.2.1 Task protocol

After the completion of the SDMT₂ trial, the angled wooden board was removed from the desk. A covered tray was then placed on the desk (as shown in Figure 6-9); care was taken to avoid making any noise which would have suggested the contents underneath the cloth or the nature of the task.



Figure 6-9. The covered tray was initially shown to participants prior to commencing the tea-making task. The red plus-sign indicates the position of the participant's gaze position.

Prior to unveiling the tray, the quality of the eye-tracking (as shown on the iViewX™ HED computer) was visually inspected by the researcher—however, there was no formal calibration process *per se* for this task other than the calibration conducted in the immediately preceding task.

Upon revealing the tray, participants were asked to “please make a cup of tea, with milk and one sugar”. The blue plastic tray contained all the items required for successful completion of the task. These included (starting from the 12-o’clock-position and moving clockwise): sugar bowl (filled with granulated white sugar to around 75% capacity), teapot (filled with lukewarm black tea to about 75% capacity), tea cup placed upside-down on a saucer with a teaspoon to its left and a milk jar (filled with trim milk to about 50% capacity).

The arrangement of the tray contents was identical for all participants (see Figure 6-10), except with the two left-handed NC participants, for whom a mirror-image arrangement was constructed instead.



Figure 6-10. Contents of the uncovered tray for the tea-making task. The red plus-sign indicates the position of the participant's gaze.

6.3.2.2 Task duration

The total duration to task completion was measured; this was regardless of the need to prompt the participant at the start or initial hesitations. The end of the task was marked by the removal of the teaspoon from the tea cup (after dissolving the sugar) and placing the teaspoon on the saucer.

6.3.2.3 Task execution

This does *not* relate to the old-age question of how the perfect cup of tea is made (as this has already been fathomed by experts in the field³). Rather, the time between fixating on the cup and the beginning of each substance's (milk, sugar and tea) pour was measured. Positive values indicate that the participant fixated on the spout until

³ Emsley, B. (2014). How to make a Perfect Cup of Tea. *News release: Royal Society of Chemistry*. Retrieved from <http://www.academiaobscura.com/wp-content/uploads/2014/10/RSC-tea-guidelines.pdf>.

after the pour began; negative values, on the other hand, designate fixating on the cup prior to pouring of the substance.

6.3.3 Results

6.3.3.1 Task duration

NC participants ($38.2 \text{ s} \pm 7.3$) completed the tea making task significantly faster ($F_{2,35} = 10.23$) than PD-N ($51.8 \text{ s} \pm 9.3$, $p < 0.001$) and PD-MCI ($50.5 \text{ s} \pm 8.3$, $p = 0.001$) participants. There was no statistically significant difference between the two PD groups.

6.3.3.2 Task execution

A significant task-effect was found upon task execution ($F_{2,35} = 10.8$, $p < 0.001$), such that fixations to the cup preceded sugar-tipping, occurred around the same time milk was poured and lagged behind pouring of the tea (see Figure 6-11). Although several PD participants ($n = 4$) were observed to exhibit slight action tremor during task execution, this did not adversely affect their performance, and no group difference was detectable among the study groups (see Figure 6-12).

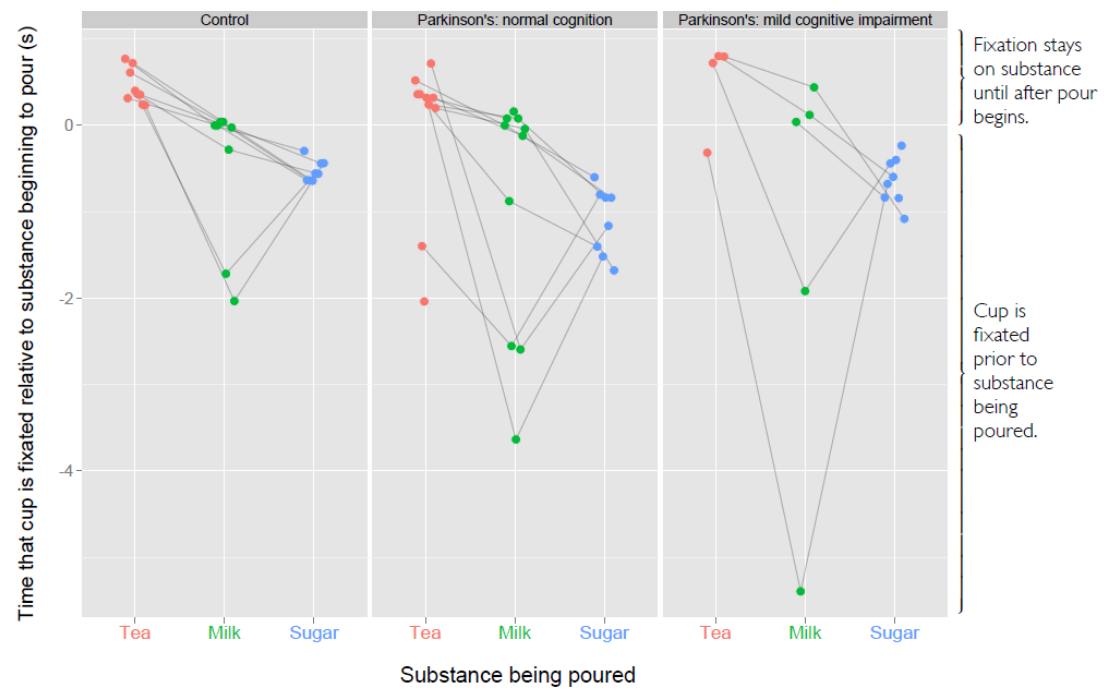


Figure 6-11. Despite a number of participants making markedly early cup fixations, the overall task execution pattern was similar among all participants (reproduced with permission from MacAskill, Alamri, Myall, Dalrymple-Alford, & Anderson, 2013).

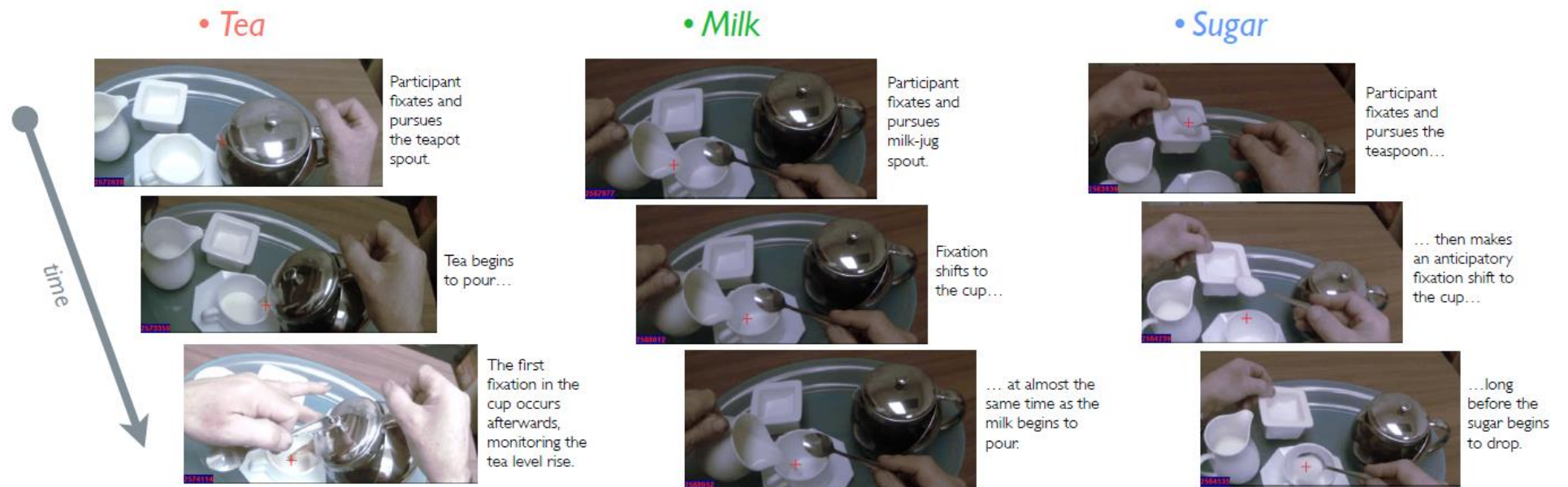


Figure 6-12. A strong task-effect is observed: cup fixations occurred prior to sugar-tipping, close to milk-pouring and after tea-pouring. This task organisation was largely preserved among NC, PD-N and PD-MCI participants. The red plus-sign indicates the position of the participant's gaze (reproduced with permission from MacAskill et al., 2013).

6.3.4 Discussion

6.3.4.1 Task duration and execution

The finding of slower task performance by PD participants was not unexpected; bradykinesia is one of the cardinal symptoms of PD (Cavanaugh et al., 2015). Given that the overall task execution strategy was similar between all participants, the longer duration to task completion is, therefore, unlikely to have been due to task organisation differences.

The rationale behind the differential, albeit consistent, fixation times vis-à-vis substance pouring remains speculative. We propose two hypotheses: the poured volume of tea is generally much larger than those of milk and sugar, and thus, a person spends more time performing this sub-task; the eyes consequently fixate on the spout until such time that the end of the pour nears, at which point a saccade is then made to the cup. A second hypothesis—although not necessarily mutually exclusive to the first—is that the person realises the more ominous consequences of spilling a hot liquid (compared with cold milk), and hence ensure a ‘closer eye’ is kept on the pouring tea.

6.3.4.2 Summary and future research

PD participants generally had preserved task organisation. Some participants (more so from the PD groups) made a few very early fixations to the next object in the task sequence. We posit this is probably in compensation for the motor slowing related to PD. Whether deficits in aspects of cognitive domains—for example, dysfunction of forward planning (Ogden, Growdon, & Crorkin, 1990)—have contributed to our findings is yet to be resolved. Inclusion of a PD-D group in the future may shed light on this.

CHAPTER 7

Concluding Remarks

7.1 Overview

The aim of this thesis research was to examine biomarkers that reflect progression during the course of the disease process in patients with PD. Findings from the different biological and behavioural markers are highlighted in the following sections.

7.1.1 Findings from the biological markers study

The blackcurrant trial was the first of its kind to investigate the presence of blackcurrant anthocyanins in human CSF. The *a priori* hypothesis was that blackcurrant anthocyanins would be detectable in plasma and possibly also in CSF. However, we demonstrated that anthocyanin species were not present in CSF (or plasma) post-oral ingestion of anthocyanin concentrate extracted from blackcurrants. The significant increase in the plasma concentration of a number of other metabolic by-products—including EGC and kaempferol—after blackcurrant supplementation has not been previously studied in *in vivo* studies.

Given the lack of blackcurrant anthocyanins in CSF, we then attempted to examine for an indirect effect of blackcurrant consumption on the CNS cellular or extracellular milieu. One way was by measuring the concentrations of IGF-1 and related molecules in CSF and plasma. We found CSF concentrations of cGP (a metabolite and regulator of IGF-1) increased significantly after blackcurrant supplementation. This is a novel but very preliminary finding. If replicated, such a finding may lead to the establishment of a unique biological marker in PD research.

We also measured CNP and NTproCNP levels in the available biofluid samples. Contrary to our hypothesis, we found PD participants had significantly lower CSF

concentrations of NTproCNP (in samples from both pre- and post- blackcurrant supplementation) compared with a normal reference range. This was also a novel finding, and was concordant with findings from stored samples obtained from the original DATATOP study (Espiner, Dalrymple-Alford, Prickett, Alamri, & Anderson, 2014). Since the reference group only reported CSF concentrations of NTproCNP (Schouten et al., 2011), we were unable to directly compare NTproCNP concentrations in plasma—a much more easily accessible biofluid. However, head-to-head comparisons of plasma concentrations of CNP and related metabolites in PD patients and healthy controls are currently underway (Z. Woodward, personal communication, December 20, 2016).

Finally, we characterised the profile of CSF and plasma exosomes in our PD participants using TRPS. Using this technique, we describe—for the first time—a tentative observation of finding the highest plasma exosome concentrations in the three participants with the lowest MoCA scores (Alamri, Vogel, MacAskill, & Anderson, 2016). Given the small number of samples, a correlation could not be statistically examined, and the result should, therefore, be interpreted with caution.

7.1.2 Key findings from the behavioural markers study

There were few differences among the study groups in performance-eye movement associations. However, we gained important insights into oculomotor behaviour during neuropsychological task completion in both healthy controls and PD patients. Such behaviour was generally similar across NC, PD-N and PD-MCI participants, with a few differences that perhaps reflect the underlying disease state as we hypothesised.

In the animal identification task, we showed that most participants, irrespective of group, tended to fixate on the animal's head in order to name it—with the areas of fixation interest being smaller for animals with 'distinctive' features (e.g., giraffe and kangaroo) than those with fewer unique features (e.g., donkey and domestic pig).

Examination of the JLO tasks revealed a higher fixation density on the distal ends of the Scanning Area lines than the proximal ends—a similar pattern to that reported of fixations upon viewing a clock face (Mosimann, Felblinger, Ballinari, Hess, & Müri, 2004). On the Where's Wally?TM Maze task, we found PD-MCI participants made significantly more vertical saccades than NC and PD-N participants. This may be the first report of such difference in directional visual pattern in PD. Whilst such difference in eye movements may be difficult to find in the clinic (i.e., as a bedside biomarker), they can be recorded in the laboratory setting (i.e., functional biomarker). Kapoula *et al* found a similar higher proportion of vertical microsaccades has been reported in AD and amnesic MCI patients (2014), although the underlying mechanism(s) of their findings may be different.

Using the mobile eye movement tracker system, we were uniquely positioned to assess eye movement behaviour in relation to the performance of dynamic tasks. On the SDMT task, we found PD-MCI participants obtained significantly lower points on the task-score than NC and PD-N participants—potentially providing a relatively easy-to-administer bedside neurocognitive marker of PD. This task-score difference was despite similar motor (i.e., UPDRS-III) and oculomotor (i.e., eye movement parameters) performance among the participants. In the tea-making task, we found a largely preserved task organisation—albeit slower to execute in PD participants—whereby fixations to the cup preceded sugar flow, occurred around the same time as milk flow and succeeded tea flow. These findings were in accordance of our hypothesis that eye movement analyses would

aid in gaining greater insight into the effect of PD on oculomotor control when patients attempt neurocognitive tasks.

7.1.3 Other findings

Significant collateral findings—not directly related to the studies' end-points—were reported throughout the thesis. The safety and efficacy of research LP (Peskind et al., 2005) was reiterated (Chapter 3). In particular, participants appreciated the repeated explanation of and reassurance prior to the procedure, which may have helped alleviate their anxiety (Alamri, MacAskill, & Anderson, 2016b). We also found our PD participants scored significantly lower on the HADS depression-subscale (Chapter 5) compared with a PD group from Saudi Arabia (Alamri, Al-Busaidi, MacAskill, & Anderson, 2015).

7.2 Critique of the biomarkers studies

7.2.1 Strengths

The behavioural markers study was designed as a case-control study, and controls were matched for age, education and sex. One of the main strengths of the behavioural markers study was the inclusion of a well-characterised group of PD-MCI participants. These were participants who satisfied the current diagnostic criteria for PD-MCI (Litvan et al., 2012).

The various components of the two studies targeted a pathophysiological aspect of PD at a certain stage of the disease process. Although there is ample literature on the need to utilise several PD biomarkers in concert, there is a paucity of studies that actually do so. This project not only included biomarkers of different *categories*

(i.e., progress and monitoring biomarkers), but also of different *types* (i.e., bedside tests, biochemical investigations and laboratory studies), as well as undergoing different analyses (i.e., both quantitative and qualitative).

7.2.2 Limitations

The low number of participants in both studies is a potential weakness. For the biological markers trials, an upper limit on the number of participants was set by the Ethics Committee due to concerns about participant safety and wellbeing in relation to the need to have repeated LP. As it happened, feedback from participants of the biological markers trials (Chapter 3) supported the safety and acceptability of repeated LP for research purposes (Alamri, MacAskill, et al., 2016b). Owing to the smaller number of enrolled participants, power analysis showed a reduction of calculated power from 0.68 to 0.47, with a subsequent increase in the chance of a Type II error.

For the behavioural markers study, effective recruitment may have been, in retrospect, stymied by two factors—restrictive inclusion criteria and disruptions after the 2011 Christchurch earthquake. Matching PD participants with each other (i.e., PD-N with PD-MCI), as well as with NC participants—for age, education and sex—severely limited the pool of participants available for the study. Moreover, in the aftermath of the earthquake in February 2011, potential Christchurch participants were probably focussed more on immediate needs (e.g., health, family, safe accommodation and getting back to work) than on volunteering to participate in the research studies. Closure of buildings for safety inspections (including the Van der Veer Clinic and the New Zealand Brain Research Institute) also prolonged the hiatus during which recruitment was not feasible.

The omission of a PD-D group in the behavioural markers study might be considered a further limitation. Recruitment of dementia patients in research can be challenging even under ideal conditions (Cooper, Ketley, & Livingston, 2014; Fargo, Carrillo, Weiner, Potter, & Khachaturian, 2016). The recruitment of sufficient numbers of local PD-D participants who satisfied the study's inclusion criteria for age, education and sex would have been challenging and likely not feasible. However, adding a group with PD-D in any future studies would provide a more picture of eye movement-performance associations in PD.

7.3 Implications and future directions

Biomarkers remain an essential element of PD research and a much-needed component of the clinical diagnosis, monitoring and management of PD patients. With substantial concerted efforts currently under way to uncover new and 're-purposed' already-available biomarkers for PD, it is difficult to foretell what the future beholds (Alamri, MacAskill, & Anderson, 2016a). Akin to the recently proposed A(myloid)/T(au)/N(euronal injury) classification of AD biomarkers (Jack et al., 2016), it may well be the case that several PD biomarkers—of different categories based upon the patho-physiological parameter(s) they measure— need to be used in combination to generate a similar descriptive scheme.

The many findings of the two studies comprising this thesis raise a number of questions. The underlying mechanism of the putative effects of berry-fruit consumption on neuronal health remains unanswered. If anthocyanins do exert a neuroprotective effect, this would need to be examined in a sizeable double-blind trial—although the absence of anthocyanins in CSF found in the present study would suggest that any such benefit might be indirect. If such putative neuroprotective effects are instead exerted by other indirect mechanisms, the

underlying mediator(s) ought to be further studied. In the case of IGF-1 and related molecules, for example, this could include examining samples from PD patients with high dietary berry-fruit intake for the interplay between dietary habits and intrathecal cGP and IGF-1—and any downstream effect(s) on the presumed IGF-1-resistance in PD.

Independent of blackcurrant anthocyanin supplementation, an obvious next step for CNP-related research is to directly compare the concentration of NTproCNP in plasma—a more easily accessible biofluid than CSF—in PD patients and matched healthy controls. If significant differences between healthy controls and PD patients are found in *plasma* concentrations of NTproCNP, this may lead to the development of a clinically useful diagnostic biomarker for PD.

Plasma exosome concentrations may be useful to compare in a large sample of PD-N, PD-MCI and PD-D patients to rigorously assess for any correlations with cognitive status. In addition, the technology utilised in the present study did not analyse exosome contents. Given the recent findings of a correlation between levels of α -synuclein in CSF-derived exosomes and the cognitive status of PD patients (Stuendl et al., 2016), examination of a similar correlation with plasma-derived exosomal contents is warranted.

Detailed quantitative analyses of the several tasks included in the behavioural markers study were largely beyond the scope of this thesis, but provide ample opportunity for future research. For instance, areas of fixation interest may be mathematically calculated in order to quantitate any differences between healthy controls and PD patients (or among well-characterised PD-N, PD-MCI and PD-D patients). Likewise, utilising eye movements, erroneous answers can be categorised and the per-group differences in each category calculated. Such further studies would permit a more detailed exploration of oculomotor

behaviour in PD, and perhaps thereby provide a greater insight into behavioural and cognitive sequelae of this condition.

Closer study of ‘problem-solving’ strategies—for example, by investigating eye movements between Scanning/Key and Working Areas—is also warranted. The effects of cultural and experiential factors could be studied more closely in relation to different neuropsychological tasks. Additional eye movement parameters, such as look-ahead fixations (Mennie, Hayhoe, & Sullivan, 2007), could be incorporated into investigations of oculomotor behaviour during natural tasks. Finally, extending this line of research to encompass other neuropsychological and natural tasks in patients with PD (of varying cognitive status) might uncover behavioural biomarkers that prove useful in the prognostication of PD—or more usefully—as biomarkers of response to new therapies.

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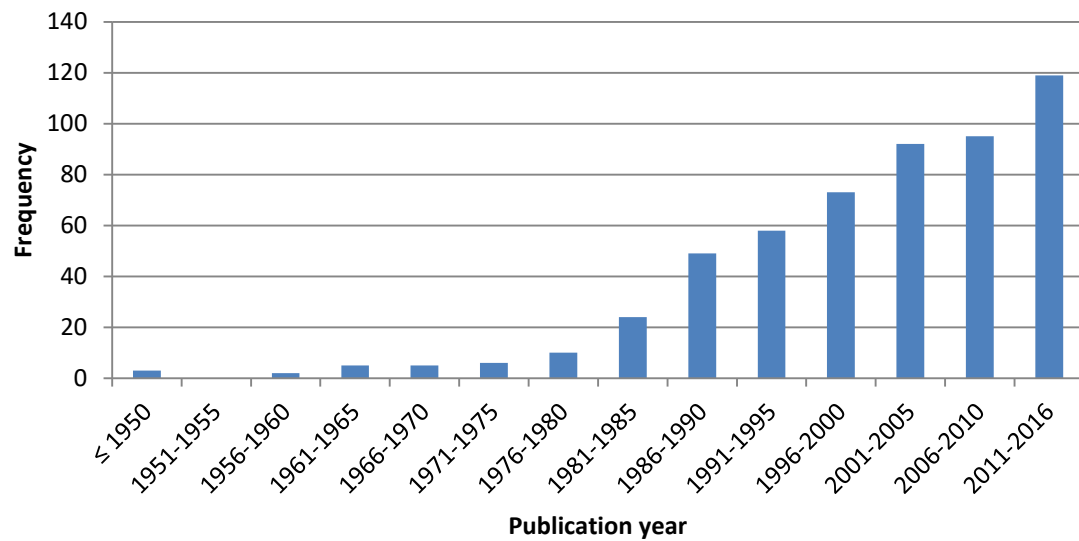
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Production notes

This thesis was written using Word 2010 and Endnote X6. Graphs were prepared in BeGaze™, Excel, R Studio and Word.

British spelling was adopted throughout this thesis, except in direct quotes and referenced items published in resources that employ the American spelling. Likewise, the possessive form of all eponymous diseases (e.g., Parkinson's disease instead of Parkinson disease) was used, with exceptions arising from direct quotes and certain references.

The serif typeface used throughout the text and figures is Palatino Linotype, other than the table of contents which is **Times New Roman**. The sans serif typeface used in the text is **Calibri**. The thesis title is Palatino Linotype.



Supplementary Figure 1. Frequency of year of publication for references cited in this thesis (n = 542). References *in press* have been included in the 2011-2016 period.